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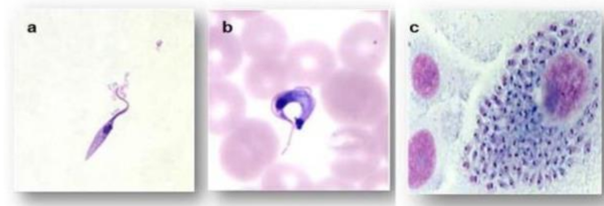
# TESIS DOCTORAL

# Estudio del diagnóstico de la enfermedad de Chagas en el Hospital Universitario y Politécnico La Fe. Desarrollo de nuevos métodos diagnósticos confirmatorios de la parasitación activa.




PROGRAMA DE DOCTORADO EN
PARASITOLOGÍA HUMANA Y ANIMAL


Presentada por

**Noelia Lozano Rodríguez**

Licenciada en Farmacia


Valencia, mayo 2025


DIRECTORES: Antonio Osuna Carrillo de Albornoz
Eva Calabuig Muñoz
María Trelis Villanueva

VNIVERSITAT DE VALÈNCIA 525 anys — Facultad de Farmacia y Ciencias de la Alimentación 50 anys

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**AUTORA: NOELIA LOZANO RODRÍGUEZ**

VNIVERSITAT DE VALÈNCIA

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Se presenta este documento de Tesis Doctoral por compendio de publicaciones para optar al título de Doctora por la Universitat de València. Se aportan para su evaluación tres artículos de investigación publicados. Los artículos científicos aquí recogidos han sido publicados en revistas científicas indexadas en el *Journal Citation Reports* de primer (Q1) o segundo (Q2) cuartil. En conjunto, adquieren una adecuada relevancia, originalidad, y excelencia, siendo la doctoranda la primera autora en todos ellos.

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# *INFORME DE LOS DIRECTORES*

Los directores, el Prof. Dr. Antonio Osuna Carrillo de Albornoz, Catedrático de Parasitología de la Universidad de Granada, la Prof. Dra. Eva Calabuig Muñoz, Profesora asociada de la Universitat de València y médico especialista en Medicina Interna, de la Unidad de Enfermedades Infecciosas del Hospital Universitario y Politécnico La Fe (Valencia) y la Prof. Dra. María Trelis, Villanueva, Profesora Titular de Parasitología de la Universitat de València y además tutora,

CERTIFICAN:

Que la Tesis Doctoral "**Estudio del diagnóstico de la enfermedad de Chagas en el Hospital Universitario y Politécnico La Fe. Desarrollo de nuevos métodos diagnósticos confirmatorios de la parasitación activa**" que se presenta para optar al grado de Doctora por la Universitat de València en la **modalidad de Tesis por compendio de publicaciones**, ha sido realizada por **Noelia Lozano Rodríguez**, N.I.F 50481844H, estudiante del Programa de Doctorado en Parasitología Humana y Animal (código 3145) y licenciada en Farmacia por la Universidad Complutense de Madrid. Reúne, a nuestro juicio, originalidad y contenidos suficientes, por lo que autorizamos su presentación para ser evaluada, emitiendo un informe FAVORABLE para la realización del depósito y defensa de la tesis doctoral.

Y para que así conste, a efectos legales, expiden el presente certificado en Valencia a

**Fdo. Antonio Osuna** **Fdo. Eva Calabuig** **Fdo. María Trelis**

“Si quieres llegar rápido camina solo, si quieres llegar lejos camina acompañado”

- Proverbio africano

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**RESUMEN**

La enfermedad de Chagas, también conocida como tripanosomiasis americana, es causada por el protozoo flagelado *Trypanosoma cruzi.* Se estima que alrededor de 8 millones de personas están infectadas, siendo endémica en 21 países de Latinoamérica. Sin embargo, debido a los continuos flujos migratorios de áreas endémicas a zonas no endémicas, actualmente se considera una enfermedad de distribución mundial. En este contexto, España juega un papel relevante, siendo el país europeo con las tasas más elevadas de la enfermedad. Una particularidad importante de la enfermedad es que, además de su trasmisión vectorial, existen otras vías de contagio, como la transmisión vertical y la transmisión a través de donantes de órgano y sangre, las cuales se dieron en España en la década de los 80. La enfermedad cursa con dos fases: una fase aguda inicial que dura unos dos meses tras la infección, con pocos síntomas patognomónicos, pero elevada parasitemia; seguida de la fase crónica, con ausencia de las formas tripomastigotas en sangre e incluso de sintomatología por un tiempo indefinido hasta que, entre el 30-40% de los pacientes, desarrollan síntomas cardíacos o digestivos.

El análisis descriptivo de los pacientes con enfermedad de Chagas del Hospital Universitario y Politécnico La Fe de Valencia, España, así como el desarrollo de nuevos métodos diagnósticos confirmatorios de la parasitemia activa mediante la obtención y separación de las vesículas extracelulares (EVs) del suero de estos pacientes, y su utilización como marcadores en la infección crónica y confirmación de la transmisión vertical, ponen en manifiesto la importancia del estudio de esta patología y sus constantes desafíos. Como resultados de la presente Tesis Doctoral, realizada por compendio de artículos, se ha analizado la prevalencia de la enfermedad, la procedencia de los pacientes, el tratamiento y las reacciones adversas de este, así como otros datos relacionados con ella.

A partir de las muestras de suero de estos pacientes, utilizando el método de ultracentrifugación como *gold* estándar y el método de concentradores de proteínas, para facilitar el procedimiento en centros con menos posibilidades tecnológicas, se han estudiado las vesículas extracelulares y los inmunocomplejos que forman con las IgGs, liberados por *T. cruzi* como marcadores para el diagnóstico y comprobación de una parasitación activa de la enfermedad. Finalmente, se confirmó que el ADN libre de células en el suero y el que se aísla de las EVs permiten detectar la parasitemia en pacientes crónicos dado que contienen ácidos nucleicos procedentes tanto de la mitocondria (ADNk) como ADN nuclear procedente de los parásitos.

Esta tesis demuestra que, ante la necesidad actual de disponer de nuevos biomarcadores que discriminen la presencia de una parasitación activa en la enfermedad de Chagas, la detección de vesículas extracelulares circulantes en estos pacientes, mediante las técnicas moleculares descritas, contribuye al avance diagnóstico y seguimiento de esta enfermedad desatendida que afecta a tantos millones de personas. Por tanto, su uso puede resultar de utilidad en diferentes situaciones de incertidumbre clínica como la fase crónica de la enfermedad, la monitorización de la eficacia del tratamiento, la sospecha de formas parasitarias quiescentes y en casos de recién nacidos, donde las pruebas tradicionales de diagnóstico no son concluyentes e imposibilitan su tratamiento precoz.

Palabras clave: Enfermedad de Chagas, vesículas extracelulares, *Trypanosoma cruzi*, diagnóstico molecular, aislamiento, parasitemia.

**SUMMARY**

Chagas disease or American trypanosomiasis, caused by the flagellated protozoan parasite *Trypanosoma cruzi*, is endemic in 21 American countries. It is estimated that about 8 million people are infected. However, we can consider it a worldwide-distributed disease due to continuous migratory movements from endemic to non-endemic regions. Spain is the non-endemic country with the highest burden of the disease in Europe. The risk lies in the fact that there are transmission pathways other than vector-born transmission; these include transplacental transmission to newborns from infected mothers or through blood or organ donations from infected individuals.

The disease has two phases: an acute phase, approximately two months post-infection, typically presents with nonspecific symptoms but is characterized by high levels of parasitemia. This is followed by a chronic phase marked by the absence of circulating trypomastigotes and often a lack of symptoms for an indeterminate period. However, 30–40% of chronically infected individuals eventually develop cardiac and/or gastrointestinal complications.

The descriptive analysis of patients with Chagas disease at the Hospital Universitario y Politécnico La Fe, Valencia, Spain, as well as the isolation and separation of extracellular vesicles (EVs) from these patients using various methods explained in this doctoral thesis, highlights the importance of studying this pathology and its constant challenges.

As part of this doctoral thesis, the prevalence of the disease, the origin of the patients, treatment regimens, and adverse reactions were analyzed, as well as other related data. Using serum samples from these patients and employing ultracentrifugation as the gold standard, as well as protein concentrators to simplify the procedure in centers with fewer technological resources, EVs and the immune complexes, released by *T. cruzi*, were

studied as markers for diagnosing and confirming active parasitosis. Finally, it was confirmed that the cell- free DNA in the serum, as well as DNA isolated from EVs, allow for the detection of parasitemia in chronic patients, as they contain nucleic acids derived from both mitochondrial (kDNA) and nuclear DNA from the parasites.

This study demonstrates that, given the current need for new biomarkers capable of distinguishing active parasitemia in Chagas disease, the detection of circulating extracellular vesicles in these patients, using the described molecular techniques, contributes to advances in the diagnosis and monitoring of this neglected disease that affects millions of people. Therefore, their use may prove useful in various clinically uncertain scenarios, such as the chronic phase of the disease, monitoring treatment efficacy, suspicion of quiescent parasitic forms, and in newborns, where traditional diagnostic tests are inconclusive and hinder early treatment.

Keywords: Chagas disease, extracellular vesicles, *Trypanosoma cruzi,* molecular diagnosis, isolation, parasitaemia.

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# SECCIÓN I: RESUMEN

# 1. Introducción

### 1.1 Enfermedad de Chagas: Epidemiología

La Enfermedad de Chagas (EC) o tripanosomiasis americana, es una enfermedad causada por el protozoo *Trypanosoma cruzi* que afecta a más de 8 millones de personas en el mundo, siendo endémica en 21 países de Latinoamérica, considerándose por parte de la OMS (2022) una de las enfermedades más importantes dentro del grupo de las enfermedades tropicales desatendidas (Pérez-Molina & Molina, 2018).

Según la Organización Mundial de la Salud (OMS) y la Organización Panamericana de la Salud (OPS) se registran unos 30.000 casos nuevos al año, aproximadamente 9.000 recién nacidos son infectados durante la gestación y hay un promedio de 12.000 muertes anuales (OPS). Hoy en día, unos 70 millones de personas siguen viviendo en zonas con riesgo de poder contraer la enfermedad.

Debido a los continuos flujos migratorios, se empezaron a detectar casos de la EC en países no endémicos, principalmente en Estados Unidos o Canadá y Europa (Figura 1). Siendo España el país con más número de casos en toda Europa, unos 55.000 estimados en 2022 (Navarro et al., 2022) y el segundo a nivel mundial, sólo EE. UU está por encima, con 300.000 casos estimados. La carga de la enfermedad en los países no endémicos cambia en función de la procedencia de la población migrante, siendo las personas de origen boliviano, ecuatoriano, argentino y colombiano las que mayores niveles de parasitación muestran. Aproximadamente encontramos un 25% de infección por *T. cruzi* en pacientes de origen boliviano, siendo esta población la más afectada en Europa (Navarro et al., 2022).

El estudio de la prevalencia de la población latinoamericana en España en los últimos años ha provocado que aumente el interés por las enfermedades procedentes de estas áreas, tanto por su implicación a nivel asistencial de los pacientes como por la necesidad de adecuar las normativas de donación para Bancos de Sangre y la prevención de la transmisión vertical en las embarazadas (Real Decreto 1088/2005, del 16 de septiembre).

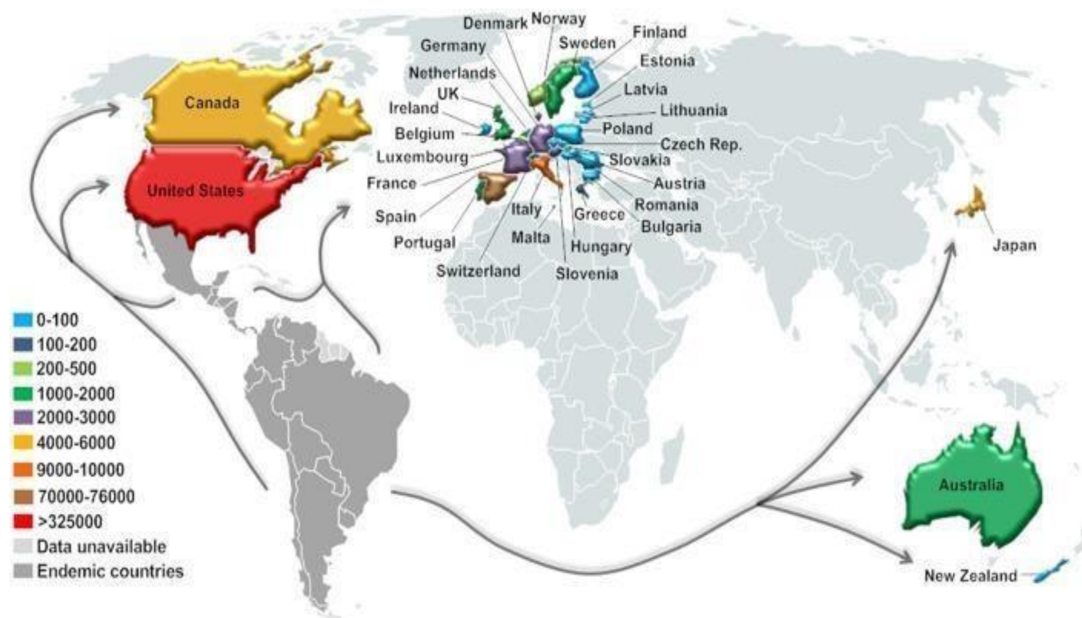



**Figura 1.** Casos estimados de migrantes con EC en los principales países no endémicos. **Fuente**: Lidani et al. (2019).

Además de la problemática sociosanitaria que provoca, y a modo de ejemplo, en 7 países de Latinoamérica causa una pérdida económica de unos 725.000 días laborales debido a muertes prematuras y 627 millones de dólares debidos a la morbilidad por el gasto que supone en la atención sanitaria (Pérez-Molina & Molina, 2018). En Estados Unidos, entre 30.000 y 45.000 personas viven actualmente con la EC de sintomatología cardíaca. Entre

2003 y 2011 se ha informado de un aumento del número de ingresos hospitalarios, la mayoría de ellos relacionados con complicación cardiovasculares graves lo que supone una gran carga económica, siendo el coste económico medio hospitalario de unos 97.903 US$ (Singh et al., 2020). Estudios realizados por Lee et al sobre los gastos de la EC muestran datos que están por encima de otro tipo de enfermedades a nivel mundial, llegando a los 9.000 millones de $ (Lee at al., 2013).

Hoy en día, sigue siendo una enfermedad subdiagnosticada donde se estima que un 82% de los pacientes siguen sin ser tratados en España y entorno a un 90% de personas con EC que viven en Europa están sin diagnosticar (Navarro et al., 2022).

## 1.2 *Trypanosoma cruzi*

*Trypanosoma cruzi* es un protozoo hemoflagelado de la familia *Trypanosomatidae*, orden *Kinetoplastida* y género *Trypanosoma* (Brener, 1973; Vallejo et al., 2009; Lidani et al., 2019). Dentro de la familia *Trypanosomatidae* hay dos géneros de gran importante clínica debido a su papel patógeno en seres humanos, *Leishmania y Trypanosoma* (Moreira et al., 2004; Kaufer et al., 2020).

Una de las características de este orden es la presencia de una estructura celular denominada kinetoplasto, que contiene material genético mitocondrial y es utilizada para poder distinguir las distintas fases del parásito (Hamilton & Stevens,2017).

- Epimastigote: es de forma flagelada, con capacidad replicativa y es la que se encuentra en el tracto digestivo del vector (Tyler & Engman, 2001).
- Tripomastigote sanguíneo: es uno de los estadios infectivos del parásito, encontrándose en el torrente circulatorio del hospedador mamífero. No tiene capacidad de división (Teixeira et al., 2012).

- Tripomastigote metacíclico: Es otro de los estadios infectivos y no replicativos del parásito. Se diferencia en el insecto vector a partir de los epimastigotes (Tyler & Engman, 2001; Teixeira et al., 2012).
- Amastigote: es de morfología redondeada. En esta fase se produce la división en el mamífero y es de localización intracelular en el hospedador mamífero (Pérez-Molina et al., 2018).

En un intento de necesidad para identificar marcadores de *T. cruzi* y distinguir diferentes subpoblaciones del parásito se empezaron a estudiar las unidades de tipificación discreta (DTUs) (Izeta-Alberdi et al., 2016). Se dividen en al menos siete, de TcI hasta TcVI y Tcbat (Velásquez-Ortiz et al., 2022). Estas DTUs suelen estar asociadas según su distribución geográfica y se utilizan para intentar comprender su riesgo de transmisión a los hospedadores mamíferos (Figura 2).

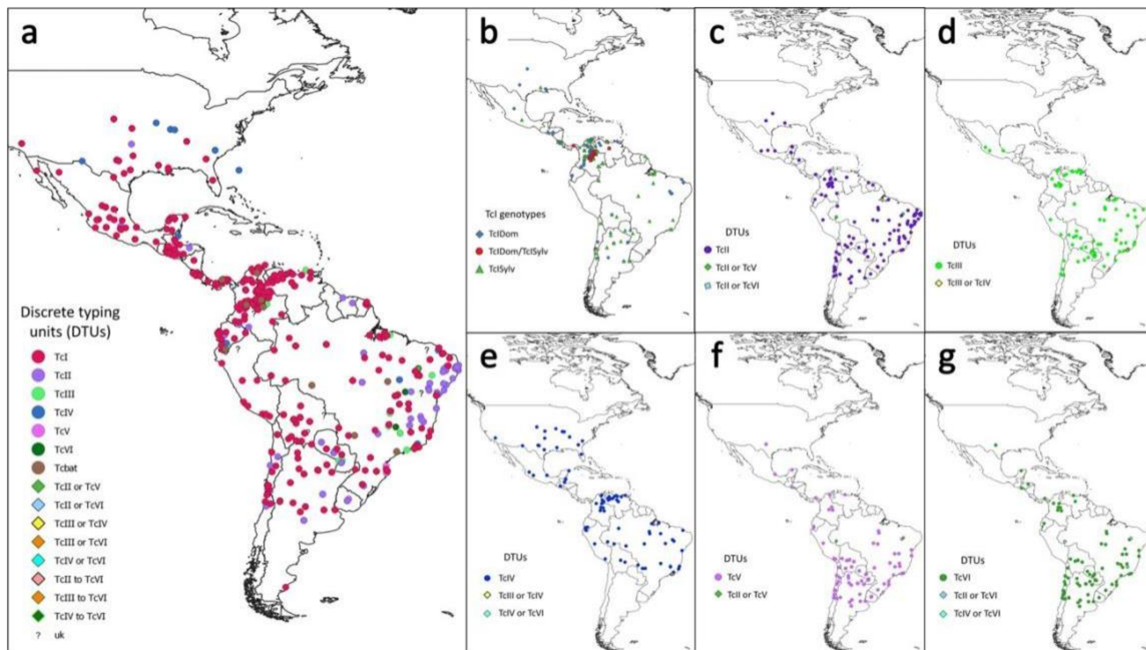



**Figura 2.** Unidades discretas de tipificación de *T. cruzi* según su distribución geográfica. **Fuente**: Velásquez-Ortiz et al. (2022).

### 1.2.1 Ciclo biológico

*Trypanosoma cruzi* presenta un complejo ciclo de vida, desarrollándose en dos ambientes diferentes, uno en el vector (triatomino) y otro en el hospedador mamífero (Figura 3).

Los insectos hematófagos pertenecientes a la familia *Triatominae* son los responsables de la trasmisión de la EC, actuando como hospedador invertebrado y vector en las áreas endémicas. Una vez que el vector ingiere la sangre del mamífero infectado, el flagelado se multiplica en el intestino medio en forma de epimastigote, llegando al final del intestino y a la ampolla rectal dando lugar a los tripomastigotes metacíclicos (formar infectivas) (Tyler & Engman, 2001; De Souza, 2002).

Cuando los insectos defecan, las formas infectivas se depositan sobre la piel y, a través de las heridas de la piel o mucosas, entran en contacto con los tejidos, invadiendo las células nucleadas. En el citoplasma de estas células se transforman en amastigotes, formas con alta capacidad replicativa, hasta que nuevamente se convierten en formas flageladas o tripomastigotes sanguíneos que son liberados a los espacios intercelulares para penetrar en las células vecinas o ingresar en el torrente sanguíneo y propagar la infección (Bern, 2015).

Las formas sanguíneas deben ser ingeridas por un nuevo vector, cerrando así el ciclo de vida natural del parásito.

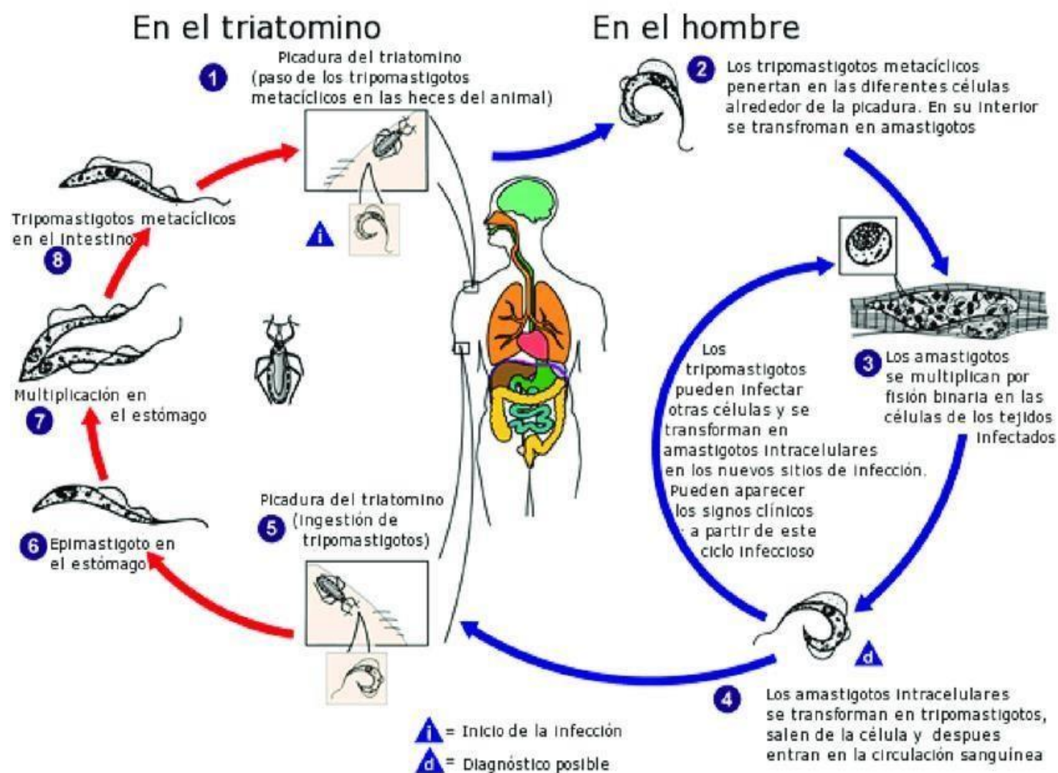



**Figura 3.** Ciclo biológico *T. cruzi.*

**Fuente:** Centers for disease Control and Prevention (CDC) (2021).

### 1.2.2 Vías de transmisión

La enfermedad de Chagas o tripanosomiais americana es una zoonosis en la que el ser humano actúa como un hospedador accidental. Existen varios mecanismos de transmisión, siendo la transmisión vectorial el más importante en áreas endémicas, pero debido a los continuos flujos migratorios, la transmisión congénita, la transfusional o por donación de órganos y la producida por accidentes de laboratorio han ido adquiriendo una elevada importancia en nuestro entorno.

Transmisión vectorial: pueden actuar como reservorio diversos mamíferos, tanto domésticos como selváticos, junto al humano y el vector, en tres ciclos de transmisión:

doméstico, peridoméstico y selvático. Es el mecanismo más frecuente en área endémica, a partir de los triatominos, conocidos por distintos nombres en función de la zona geográfica (vinchuca, chinche besucona, barbeiro…). Existen más de 140 especies capaces de trasmitir la EC (Coura, 2015).

Transmisión oral: se produce a partir de alimentos o bebidas contaminadas con heces o triturados de los insectos triatominos. Si bien en algunos casos existe la sospecha de contaminación con orina de Zarigüeyas infectadas donde en la orina de estos marsupiales se diferencia el *Trypanosoma* en formas metacíclicas. La mayoría de estos casos se presentan como brotes de infección aguda grave, con alta carga parasitaria. Las bebidas populares como el jugo de caña, maracuyá o de frutas anonáceas, están asociados a un gran número de brotes en varios países (Alarcón de Noya et al., 2010; Díaz & González, 2014).

Transmisión congénita: puede ocurrir alrededor de un 5% de los recién nacidos de madres con EC (Carlier et al., 2015), siendo el principal mecanismo de transmisión en áreas no endémicas. Gracias a las campañas para reducir la transmisión vectorial, la transmisión vertical se ha convertido en una de las principales vías de la EC en todo el mundo, siendo el principal objetivo de prevención (OMS, 2015). El proceso en el que ocurre no se conoce completamente ya que se ven implicados diferentes factores, relaciones con las condiciones de inmunosupresión en la gestación (Hermann et al., 2014), las características fetales (Torrico et al., 2005), la parasitema o la UDT que condiciona su histotropismo por la placenta (Carlier et al., 2015; Kemmerling et al., 2019).

La parasitemia detectada en el tercer trimestre de la gestación es el factor de riesgo que más se ha asociado con la probabilidad de que se produzca la transmisión madre-hijo

(Brutus et al., 2010; Murcia et al., 2013), pudiéndose dar en cualquier estadio de la infección, tanto en la fase aguda como crónica (Torrico et al., 2004).

En España, se han tomado algunas medidas de cribado, tales como el diagnostico serológico o molecular, de todas las mujeres embarazadas procedentes de regiones endémicas o en aquellas donde las madres proceden de dichas regiones. En la región de Murcia las medidas adoptadas por su servicio de salud han sido realizar un análisis serológico de *T. cruzi* a las gestantes, tanto si proceden de Latinoamérica, como si no. No existen evidencias que confirmen la transmisión a través de la leche materna, por lo que no se recomienda la interrupción de la lactancia, salvo en los casos que la madre se encuentre en la fase aguda o que presente heridas en los pezones (Bittencourt 1992; Rassi et al., 2004).

Transmisión transfusional: Este mecanismo fue considerado uno de los principales mecanismos de transmisión de la EC, especialmente en países donde la transmisión vectorial no puede tener lugar como los países de la UE, al no existir los vectores que participan en la misma. La detección de algunos casos de transmisión transfusional en España dio lugar a que en 2005 se implantaran las medidas a seguir en caso de donantes potencialmente transmisores de la EC (Real Decreto 1088/2005). Según este decreto, es de obligado cumplimiento en todos los centros de transfusión españoles la realización de estudios serológicos a todo donante procedente de área endémica o nacido de madre procedente de área endémica. Dentro de la UE, fue España el primer país en aplicar esta norma, siendo referente a nivel mundial, dado que se tomaron medidas que aún no existían en gran parte de los países endémicos del continente americano.

Transmisión por donación de órganos: El cribado es obligatorio a todo donante procedente de área endémica o nacido de madre procedente de área endémica, estando contraindicado cuando el donante presenta una infección en fase aguda o cuando los órganos implicados son el corazón y el intestino (Len et al., 2007). El riesgo de transmisión depende del órgano trasplantado: corazón (75%), pulmón (66,6%), hígado (29%) o el riñón (18%) (Hidron et al., 2010). En el caso de los receptores negativos que reciben un órgano de un donante seropositivo, se debe monitorizar un seguimiento, ya que existe la posibilidad de que se produzca una transmisión post-trasplante.

## 1.3 Manifestaciones clínicas

### 1.3.1 Fase aguda

La fase aguda de la EC tiene una duración variable de unos dos a tres meses desde la exposición al parásito. Esta fase se puede diagnosticar mediante visualización directa y normalmente cursa de manera asintomática, donde tan sólo el 5% de las personas presentan alguna sintomatología, considerada como leve y poco patognomónica. Estas manifestaciones pueden ser leves, desde un malestar general pudiendo observar el signo de Romaña o un edema palpebral cuando la picadura ocurre en el ojo, hasta causar hepatoesplenomegalia, meningitis y miocarditis en los casos más graves (Rassi et al., 2010; Pérez-Molina & Molina, 2018).

Estas manifestaciones oculares se suelen observar, casi exclusivamente, en zonas de áreas endémicas. En España esta fase la podemos observar en los primeros meses de vida de los recién nacidos, así como en casos de transfusión de sangre, trasplante de órganos y accidentes de laboratorio.

### 1.2.2. Fase crónica

Una vez pasados unos meses, la enfermedad entra en una fase crónica, donde encontramos niveles de parasitemia muy bajos en sangre gracias al control que produce el sistema inmunitario y la subsiguiente elevación de Inmunoglobulinas (IgGs) (Figura 4). El diagnóstico en esta fase debe realizarse por métodos serológicos para la determinación de anticuerpos frente al parásito. Entorno a un 60-70% de las personas pueden mantenerse asintomáticos toda la vida. Pero, aún por causas desconocidas, un 30-40% de las personas infectadas pueden llegar a desarrollar alteraciones cardíacas, digestivas o del sistema nervioso central. Normalmente estos síntomas suelen aparecer entre los 10 y 30 años de contraer la enfermedad (Pérez-Molina & Molina, 2018).

La mayoría (60-70%) de los pacientes no tratados entran en la fase crónica y se mantienen asintomáticos durante años. Esta fase es conocida como crónica indeterminada. Dentro de los pacientes que entran en la fase crónica sintomática, unos 20-30% desarrollan una afectación cardíaca, que se caracterizan por presentar un cuadro de disnea, palpitaciones, mareo, edemas en miembros inferiores, arritmias e insuficiencia cardíaca. Una combinación típica del electrocardiograma (ECG) es el bloqueo rama derecha (BRDHH) y hemibloqueo anterior izquierdo (HBAI) (Gascón et al., 2007). La principal causa de muerte es la taquicardia ventricular (TV) y la muerte súbita, seguido de la insuficiencia cardíaca (IC) y otros eventos tromboembólicos (Maguire et al., 1982).

Por otro lado, entre el 10-15% de los pacientes pueden presentar una afectación digestiva, (Pérez-Ayala et al., 2011; Pérez-Molina & Molina, 2018) y suelen ser características las afectaciones de esófago y colon. La desaparición progresiva del sistema nervioso entérico está detrás de los desórdenes de la motilidad intestinal y de la dilatación del tracto

digestivo, apareciendo el megaesófago, disfagia, dolor retroesternal, reflujo, malabsorción, megacolon y estreñimiento grave (Sánchez-Montalvá et al., 2016).

Dentro de los pacientes con una miocardiopatía, entre el 5-20% pueden presentar afectación esofágica (Pérez-Molina & Molina, 2018).

Según la región y el momento del estudio podemos encontrar niveles de síntomas más o menos elevados en recién nacidos de madres con EC. Aunque históricamente las tasas de morbimortalidad en recién nacidos eran elevadas (Torrico et al., 2004; Carlier et al., 2019), hoy en día la mayoría de estos niños y niñas no presentan sintomatología característica de la EC (Carlier et al., 2019).

Cuando encontramos la EC en una gestante, es común la presencia de partos prematuros, lesiones en los órganos del feto y abortos. A las horas o días del nacimiento pueden detectarse síntomas más graves (hepatoesplenomegalia, distrés respiratorio, miocarditis, ictericia, alteraciones cardíacas e incluso meningoencefalitis) (Cevallos & Hernández, 2014).

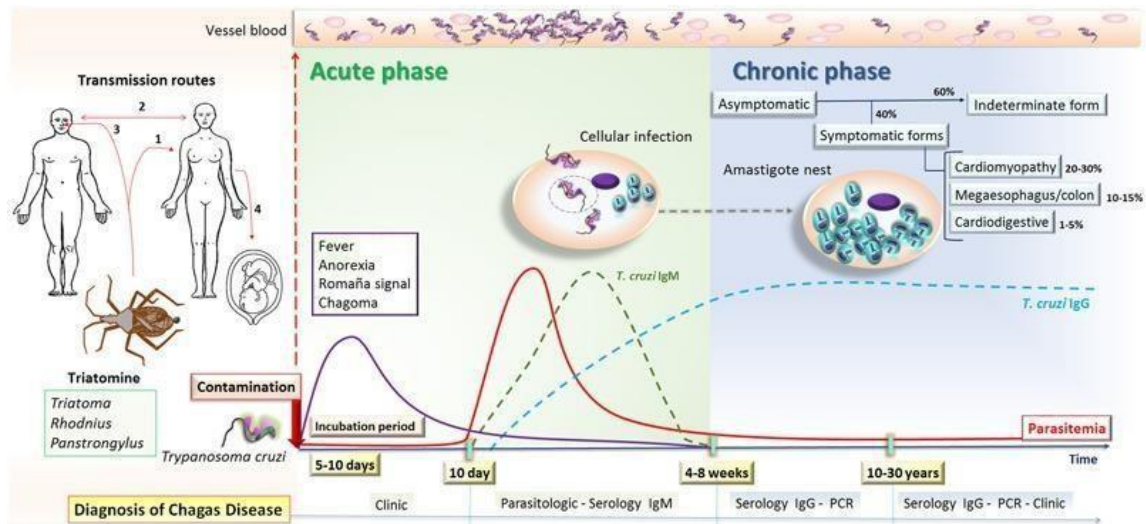



**Figura 4.** Curso natural de la infección por *T. cruzi* en humanos.

**Fuente**: Lidani et al. (2017).

## 1.4 Diagnóstico

El diagnóstico de la EC es fundamentalmente de laboratorio, dado que muchos pacientes permanecen asintomáticos durante toda su vida.

### 1.4.1 Laboratorio

#### 1.4.1.1 Parasitológico

El diagnóstico directo de la EC está indicado en la fase aguda y se basa en la observación del parásito por microscopía óptica de la sangre del paciente. Esta observación microscópica puede realizarse en fresco o mediante tinción de Giemsa de gota gruesa o de frotis sanguíneo (Feilij et al., 1983).

Otros métodos directos son el xenodiagnóstico, consiste en la aplicación de triatominos de "laboratorio" en el brazo o vientre del paciente y examinar mensualmente la presencia de tripomastigotes en el contenido rectal del triatomino.

Otra técnica es el método de Strout que consiste en la concentración de los parásitos a partir de sangre sin anticoagulante. Así, los tripomastigotes se quedan suspendidos en el sobrenadante. Por último, el método del microhematocrito o tubo capilar heparinizado permite observar al microscopio el movimiento de los parásitos en la interfase entre los hematíes y la capa de leucocitos (Feilij et al., 1983; La Fuente et al., 1984).

#### 1.4.1.2 Diagnóstico Molecular

Esta técnica ha cobrado gran interés en el diagnóstico de la EC del recién nacido cuando exista la sospecha de transmisión vertical y en los casos de posible reactivación en pacientes inmunodeprimidos (Schijman, 2018). También se ha demostrado que tiene gran utilidad en el seguimiento post-tratamiento, tanto de niños como de adultos (Murcia et al., 2010; Schijman, 2018) a pesar de que la sensibilidad de la detección de ADN del parásito

está entre el 50 y el 70% en la fase crónica de la enfermedad, ya que la parasitemia en esta fase es fluctuante.

Existen varias secuencias dianas del parásito para la detección molecular. Las más utilizadas son la amplificación del ADN mitocondrial y en concreto el ADN de los minicírculos del kinetoplasto de *T. cruzi* (ADNk), que contiene entre 10.000 y 30.000 minicírculos, y cada uno de estos presenta cuatro copias de la región variable, llegando a encontrar hasta 120.000 copias del parásito. Otros marcador molecular, ampliamente usado para la amplificación y diagnóstico de la EC, es el ADN satélite (ADNsat) de *T. cruzi,* formado por 120.000 copias aproximadamente, representando el 10% del parásito presente en el núcleo del protozoo. La sensibilidad de PCR que amplifica ADNk es mayor que la de ADNsat, mientras que esta última presenta mayor especificidad (Gomes et al., 1998; Schijman et al., 2011; Ferrer, 2015).

El uso de PCR a tiempo real cuantitativa (qPCR) nos permite conocer la carga parasitaria, permitiendo un posible control post-tratamiento en los pacientes en fase crónica (Simón et al., 2020). A pesar de su gran uso, hoy en día no se dispone de protocolos de PCR estandarizados para su correcto uso en todos los hospitales y centros de diagnóstico (Ferrer, 2015). Como puntos críticos para mejorar la eficacia en el diagnostico por PCR, se encuentran el volumen de sangre extraída, el tratamiento o no con agentes caotrópicos como la guanidina y los procedimientos de extracción de los ácidos nucleicos, siendo el más recomendable el método de extracción no automatizado como es la extracción con fenol/cloroformo/ alcohol isoamílico (Schijman, 2018).

#### 1.4.1.3 Serológico

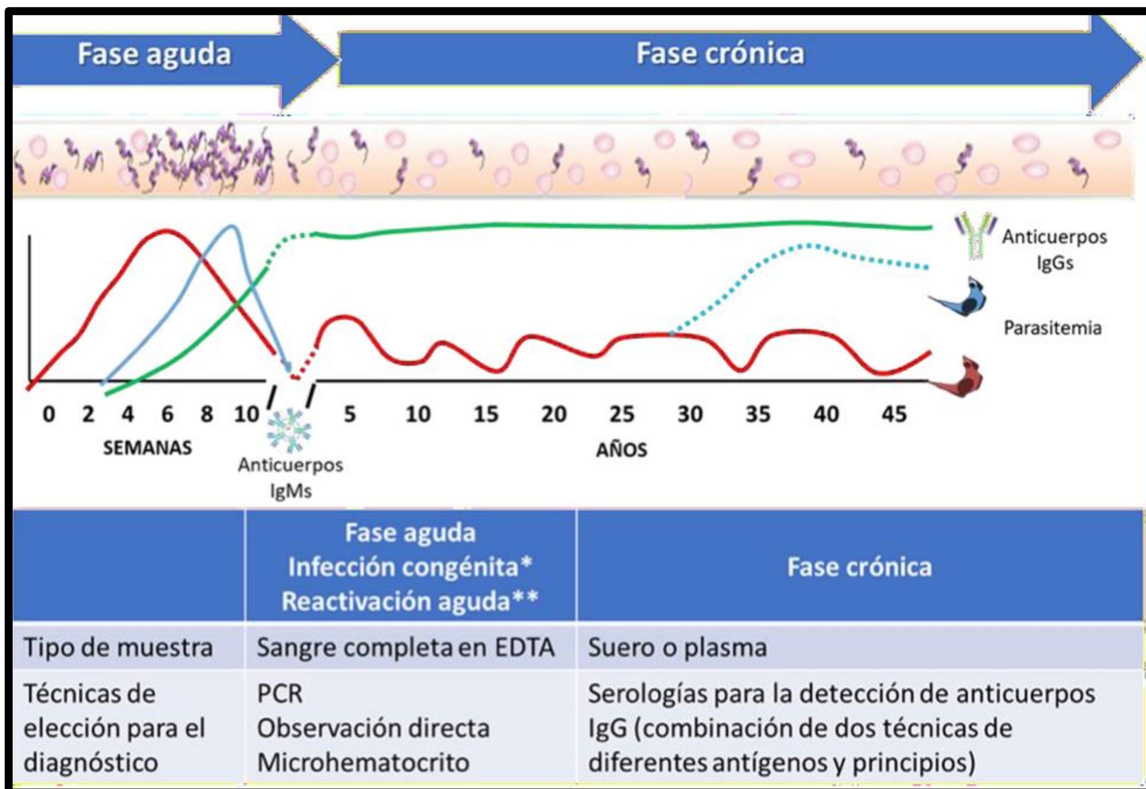
La detección de anticuerpos frente a *T. cruzi* se utiliza para el diagnóstico de la EC, tanto al final de la fase aguda, como en la fase crónica (Figura 5), donde la respuesta inmunológica frente al parásito es elevada y los niveles de parasitemia son bajos (Gabaldón-Figueira et al., 2023). Dado que los niveles de IgG se mantienen elevados durante toda la vida, aun después de los tratamientos, indican que el paciente ha estado infectado, pero no determinan la presencia de una parasitación activa. En función de la diversidad de los resultados inmunológicos, muchos de ellos no determinantes y consecuencia de las diferentes cepas, los antígenos empleados e incluso la variabilidad geográfica, ha provocado que, tanto la PAO como la OMS, recomienden confirmar el diagnóstico mediante el uso de al menos dos técnicas serológicas que utilicen diferentes antígenos, de cepas de parásitos, con diversas DTUs y de origen geográfico diferente (WHO, 2002).

Existen dos tipos de técnicas serológicas en función del tipo de antígeno que se utilice. Las técnicas que emplean antígenos nativos y las que utilizan antígenos recombinantes o péptidos sintéticos (Abras et al., 2016) Su objetivo es llegar a alcanzar la sensibilidad y especificidad necesaria (da Silveira et al., 2001; Caballero et al., 2007) y que den resultados coherentes y sin discrepancias. Entre las técnicas utilizadas en los diferentes laboratorios se encuentran:

- ELISA (Enzyme-Linked ImmunoSorbent Assay): es una de las técnicas más utilizada que emplea tanto parásitos completos como fracciones antigénicas o incluso antígenos recombinantes o sintéticos adsorbidos sobre las placas de microtitulación. En el caso de usar parásitos completos o lisados, esto puede provocar reacciones cruzadas frente a otras enfermedades infecciosas como en el

caso de la leishmaniasis o *Trypanosoma rangeli* (Caballero et al., 2007; Gabaldón-Figueira., 2023).

- IFI (InmunoFluorescencia Indirecta): los anticuerpos presentes en el suero del paciente son colocados sobre una lámina que contiene el antígeno, parásitos fijados, y son revelados a través de anticuerpos anti-inmunoglobulina humanos unidos a fluoresceína (Caballero et al., 2007; Gabaldón-Figueira., 2023). Tiene el inconveniente de las reacciones cruzadas como se ha indicado previamente. Actualmente se usa como técnica confirmatoria cuando otras técnicas han dado resultados con incongruencias.
- HAI (HemAglutinación indirecta): consiste en la reacción antígeno-anticuerpo que producen los glóbulos rojos sensibilizados con antígenos de *T. cruzi* y en presencia de los anticuerpos contra el parásito. Es una reacción que da lugar a la aglutinación de los eritrocitos (Caballero et al., 2007; Gabaldón-Figueira., 2023).
- IC (InmunoCromatografías): Pueden existir en diferentes formatos y emplean antígenos purificados, recombinantes o péptidos sintéticos. El resultado se obtiene en unos 15 minutos, se necesita poca cantidad de sangre y no requiere personal entrenado para su realización.
- CMIA (Inmunoensayo quimioluminiscente de micropartículas): se ha desarrollado en los últimos años y es una técnica automatizada basada en la quimioluminiscencia, empleando antígenos recombinantes con gran número de fracciones antigénicas y cepas. Es la técnica empleada hoy en día para el cribado dada su gran sensibilidad y especificidad (Flores-Chavez et al., 2018), planteándose así, un firme candidato como método para el cribado de la EC (Abras et al., 2020).




| | Fase aguda<br>Infección congénita*<br>Reactivación aguda** | Fase crónica |
|---|---|---|
| Tipo de muestra | Sangre completa en EDTA | Suero o plasma |
| Técnicas de elección para el diagnóstico | PCR<br>Observación directa<br>Microhematocrito | Serologías para la detección de anticuerpos IgG (combinación de dos técnicas de diferentes antígenos y principios) |

**Figura 5.** Diagnóstico de la enfermedad de Chagas. EDTA: ácido etilenaminotatraacético; PCR: Polimerase Chain Reaction; IgG: Inmunoglobulina G; IgM: Inmunoglobulina M.

**Fuente**: Sulleiro et al. (2021).

### 1.4.2 Clínico: complicaciones de la enfermedad de Chagas crónica

Para el diagnóstico de las complicaciones por la EC, ocurridas en la fase crónica de la enfermedad, existen diferentes valoraciones en función de la afectación de cada paciente. Para poder estratificar la afectación cardíaca existen numerosas clasificaciones. Todas ellas ponen en valor la conducción cardíaca, la estructura cardíaca y los síntomas del paciente (Kuschnir et al., 1985; Viotti et al., 2004).

A nivel clínico, las alteraciones cardíacas se pueden observan realizando diferentes técnicas:

- Electrocardiograma (ECG): se recomienda realizar a todos los pacientes en cualquier fase de la enfermedad. Las alteraciones electrocardiográficas que podemos encontrar son hemibloqueos anterior o posterior, bloqueo de rama

derecha, bloqueo de rama izquierda, extrasístole ventricular, bloqueos auriculoventriculares, alteraciones del ST, presencia de onda Q anómala, bradicardia, bajo voltaje de QRS, arritmias auriculares, taquicardias ventriculares e incluso la muerte súbita (Maguire at al., 1983).

- Radiografía de tórax: se utiliza para valorar la presencia de cardiomegalia.
- Ecocardiograma: se realiza para evaluar alteraciones mecánicas precoces, las alteraciones segmentarias de la contractilidad miocárdica y el aumento del diámetro telediastólico del ventrículo izquierdo, que son las principales alteraciones de la cardiopatía chagásica (Acquatella, 2007).

Los episodios tromboembólicos también tienen especial importancia en la EC crónica, tanto a nivel sistémico como pulmonar, considerando la profilaxis con terapia anticoagulante (Braga et al., 1995). Otras alteraciones cardíacas estructurales que ocurren en la EC crónica van desde zonas fibróticas o aneurismas apicales hasta la miocardiopatía dilatada.

Existen varias clasificaciones para estratificar a estos pacientes. Una de las más conocidas es la de Kuschnir, que se basa en la radiografía, el electrocardiograma y los síntomas del paciente. Otras clasificaciones más actuales ya introducen otras técnicas como el ecocardiograma, la prueba de esfuerzo o la monitorización electrocardiográfica durante 24 horas (Andrade et al., 2011).

Por otro lado, las complicaciones digestivas de la EC suelen afectar principalmente al colon y al esófago, aunque pueden llegar a localizarse en cualquier tramo del tubo digestivo (Iantorno et al., 2007; Matsuda et al., 2009). Las pruebas que se realizan para observar este tipo de complicaciones son el esofagograma, el tránsito intestinal con contraste y el enema opaco.

## 1.5 Tratamiento

Los dos únicos fármacos utilizados para el tratamiento de la EC son el benznidazol (BNZ) desde 1971 y el nifurtimox (NFX) desde 1965 (WHO,2002; Bern et al., 2007). En España son medicamentos que no están disponibles, por lo que se deben solicitar de forma individualizada como medicación extranjera.

La dosis recomendada del BNZ en adultos es de 5 a 7 mg/kg/día distribuido en 2 o 3 tomas durante 60 días y en niños (1-10 años) es de 10 mg/kg peso/día repartido en 2 dosis durante 60 días. La dosis de NFX recomendada para adultos es de 8 a 10 mg/kg, para adolescentes es de 12,5 a 15 mg/kg y para los niños de 1 a 10 años es de 15 a 20 mg/kg. Se administra por vía oral repartido en 4 tomas durante 90 a 120 días.

Una de las limitaciones del tratamiento de la EC es la alta tasa de efectos adversos relacionados con el uso de estos dos medicamentos. Esto ocurre hasta en un 40% en los pacientes con BNZ y hasta un 61% en los tratados con NFX, causando el abandono del tratamiento en un alto porcentaje de pacientes (Carrilero et al., 2011; Murcia et al., 2012). Este es uno de los motivos por lo que el BNZ es el tratamiento de elección, ya que presenta menos efectos adversos que el NFX, aunque la tolerancia puede ser muy variable entre pacientes (Bern et al., 2007).

Los efectos adversos asociados al BNZ más comunes suelen ser de tipo dermatológico, que ocurren en un tercio de los pacientes, apareciendo reacciones cutáneas más o menos grave. También puede cursar con neuropatía periférica y granulocitopenia. En algunas ocasiones presentan eosinofilia, produciendo un síndrome de DRESS (*Drug Reaction with Eosinophilia and Systemic Symptoms* o reacción de sensibilidad a medicamentos con eosinofilia y síntomas sistémicos) (Molina et al., 2015).

Los efectos adversos más frecuentes relacionados con el NFX son gastrointestinales (Bern et al., 2007).

El tratamiento precoz en la EC congénita presenta tasas de curación cercanas al 100% (Galvao et al., 2003; Altcheh et al., 2010; Carlier et al., 2011). Ambos fármacos están indicados en la fase aguda de la enfermedad, mientras que en la fase crónica de la enfermedad no está muy demostrada su eficacia.

Podemos observar también situaciones de reactivación de formas inactivas en pacientes inmunocomprometidos dando lugar a nuevos signos y síntomas de la EC (Hemmige et al., 2012). Al igual que ocurre en otros protozoos como *Leishmania* spp. y *Toxoplasma gondii,* en *T. cruzi* se desarrollan formas inactivas latentes que contribuyen al establecimiento de infecciones crónicas y que son refractarias a los tratamientos farmacológicos (Barrett et al., 2019).

El consenso actual es tratar a toda persona con EC hasta los 18 años, ofrecer el tratamiento a los adultos hasta 50 años que no presenten afectación visceral avanzada y opcionalmente a los mayores de 50 años (Bern et al., 2007), así como a toda mujer en edad fértil procedente de área endémica para intentar prevenir la trasmisión vertical (Murcia et al., 2012).

## 1.6 Vesículas extracelulares

Las vesículas extracelulares (EVs) son pequeñas vesículas de membrana que se pueden clasificar según su tamaño, biogénesis o ruta en que se liberen al medio extracelular; exosomas (30-150 nm), ectosomas (100-1000nm) y cuerpos apoptóticos (>1µm). Son portadoras de una amplia variedad de lípidos, proteínas y diferentes poblaciones de ARN, ADNss, proteínas que participan en procesos metabólicos, proteínas del citoesqueleto,

antígenos del complejo mayor de histocompatibilidad de clase I y II, proteínas ligadas a balsas lipídicas y una serie de proteínas de membrana ancladas a la misma como son las tetraspaninas, entre otras (De Sousa et al., 2022).

El gran interés que despiertan las EVs es su participación en diferentes tipos de patologías, como cáncer (Bebelman et al., 2018), enfermedades autoinmunes (Antwi-Baffour et al., 2010), infecciones (Antwi-Baffour et al., 2019; Rossi et al., 2021; De Sousa et al., 2022) y enfermedades neurodegenerativas (Lange et al., 2017; Xiao et al., 2021). Participan en la comunicación celular, la interacción patógeno-célula hospedadora, la modulación de los nichos celulares (como es el caso de tumores) y la modulación de la respuesta inmunitaria (van der Pol et al., 2012; Thèry et al., 2018).

### 1.6.1 Biogénesis

Las EVs se generan por un proceso activo dependiente de ATP, gracias a un proceso que implica una doble invaginación de la membrana plasmática y la formación de cuerpos multivesiculares intracelulares (Multivesicular bodies, MVB) que contienen vesículas intraluminales (Intralumenal vesicles, ILV).

La primera invaginación de la membrana plasmática forma una estructura en forma de copa que incluye proteínas de la superficie celular y proteínas solubles asociadas con el medio extracelular. Esto produce la formación de un endosoma temprano que, en algunas ocasiones, puede fusionarse directamente con un endosoma preexistente y con material procedente del retículo endoplasmático y el aparato de Golgi. Estos endosomas pueden madurar o dirigirse hacia la fusión con los lisosomas o eventualmente generar MVB.

Los MVB se forman por invaginación de la membrana limitante endosómica (doble invaginación de la membrana plasmática). Este proceso produce como resultado cuerpos multivesiculares que contienen vesículas intraluminales (futuros exosomas). Los cuerpos multivesiculares pueden fusionarse con lisosomas o autofagosomas para degradarse o fusionarse con la membrana plasmática para liberar las ILV contenidas como exosomas (Kalluri & LeBleu, 2020) (Figura 6). En todo este proceso de formación intervienen una serie de complejos enzimáticos como el denominado complejo ESCRT-0, -I, -II, -III; la ATPasa VPS4 (vacuolar protein sorting 4). En la formación vesículas, como ya se ha indicado en las EVs existen diferentes proteínas como la Ras GTPasa Rab, TSG101, sindecan-1, ESCRT (complejos necesarios para el transporte), fosfolípidos, ceramidas, esfingomielinasas y SNARE (receptor de proteína de unión del factor sensible a N-etilmaleimida soluble (NSF)) que están involucrados en el origen y proceso de biogénesis de los exosomas (Bebelman et al., 2018)

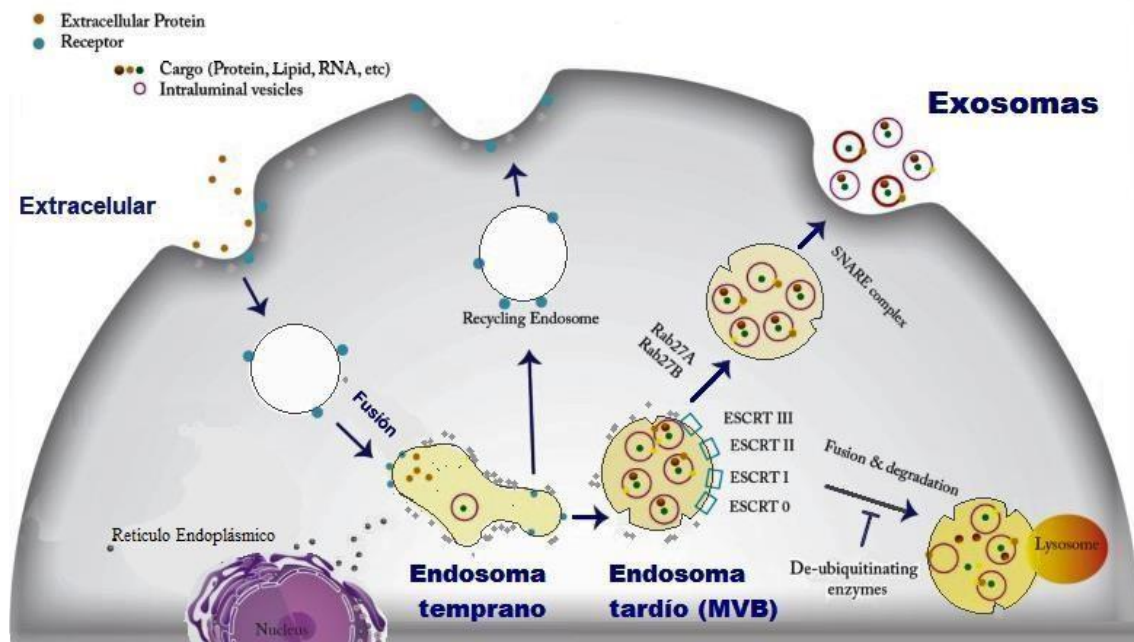



**Figura 6.** Biogénesis del exosoma y secreción dentro del sistema endosomal.
**Fuente** Mashouri et al. (2019).

### 1.6.2 Composición molecular

Para poder averiguar la composición de las distintas EVs, se han utilizado diferentes técnicas: *western blotting*, inmune-ME, sorting de células estimuladas por fluorescencia y espectrometría de masas. Debido a que cada EVs va a tener diferente composición molecular, dependiendo de la célula que proceda, se ha elaborado una base de datos denominada Exocarta. Una base con resultados de células de mamíferos y otra para EVs no mamíferas en la que se encuentran proteínas, lípidos y ácidos nucleicos.

Respecto a la composición proteica, las primeras proteínas que forman parte son las relacionadas en su biogénesis, clatrina y chaperonas. Otra familia importante son las Tetraspaninas (CD63, CD9, CD53, CD37, CD 81 Y CD82). Algunos tipos de células presentan moléculas únicas en sus exosomas como es el caso de CD86 y MHCII. Otro tipo de proteínas encontradas son las enzimas como la enolasa, aldolasa, ATP, citrato liasa, etc., además de proteínas encargadas en la transducción de señales (quinasas) y proteínas del citoesqueleto encargadas del trasporte de estas vesículas como la actina, tubulina, miosina entre otras. También se han encontrado histonas, proteínas ribosomales y de adhesión como las integrinas.

En cuanto la composición lipídica, las EVs están formadas por una bicapa lipídica similar a la de la membrana plasmática. El colesterol, los esfingolípidos y los fosfatidilglicéridos son algunos de ellos.

En 2011, Batista et al., describieron un patrón de glicosilación conservado en los exosomas de células T, célula de melanoma, células de cáncer de colon y en exosomas presentes en leche humana. Se caracterizan por tener alto contenido en N-glicanos, manosa, ácido siálico y polilactosamina A. El papel del glicoma de las EVs se ha relacionado con la invasión tumoral.

Los componentes más importantes con respecto a la comunicación intercelular son los mARNs y miARN, encontrados en el interior de las EVs. Están involucrados en el silenciamiento post- transcripcional de los genes y actuarían en la patogénesis de muchas enfermedades. Por lo que el estudio de la composición molecular de las EVs es de especial interés para el diagnóstico de diferentes enfermedades producidas en el ser humano.

El estudio proteómico de las EVs de *T. cruzi* procedente de las formas epimastigotas y tripomastigotas infectantes fue llevado a cabo por Renata et al. (2021).

### 1.6.3 EVs en enfermedades infecciosas

En los últimos años, las EVs se han estudiado en diversas patologías, observando que juegan un papel importante en la infectividad y el posterior desarrollo de la enfermedad. El uso de las EVs se ha empleado en numerosos estudios para el diagnóstico tumoral, sin embargo, se ha visto que también tienen especial interés en patologías producidas por agentes infecciosos.

En la infección por el virus de la inmunodeficiencia humana (VIH) se ha visto que las EVs intervienen en la transferencia de proteínas y del ARN. En las bacterias gram negativas participan en la comunicación con otras células y poseen factores de virulencia que utilizan para la unión y la invasión de células. Y respecto a las enfermedades producidas por parásitos, se ha comprobado el gran papel que desempeñan en el parásito para provocar daño a su hospedador. Se han mostrado como otro componente de los productos de secreción del parásito para su absorción e integración en la célula. Se forman como instrumentos de reorganización estructural, respuesta al estrés y supervivencia (Deatheragea & Cookson, 2012). La infección por *Plasmodium* spp. y *Leishmania* spp. son algunos de los ejemplos.

Estudios recientes han sugerido que las vesículas extracelulares pueden vehiculizar moléculas a las células del hospedador y modular la respuesta inmunitaria del hospedador (Samoil et al., 2018; Cornet-Gómez et al., 2023).

### 1.6.4 EVs en *Trypanosoma cruzi*

Las EVs de *T. cruzi* forman parte de su secretoma, junto con todas las proteínas que secretan al medio extracelular por otras vías.

La mayoría de los estudios realizados en diferentes modelos de tripanosomátidos, muestran que las EVs transportan proteínas y material genético relacionado con la virulencia del parásito (Bautista-López et al., 2017; Rossi et al., 2021).

Muchos grupos han intentado conocer el papel que tienen estas EVs en la EC, averiguando que la liberación de las EVs por los MVB se produce a través de la bolsa del flagelo y ocurre en todas las etapas del desarrollo del parásito (De Pablos et al., 2016; Díaz Lozano et al., 2017).

Los epimastigotes y los tripomastigotes liberan dos clases de EVs que incluyen ectosomas y vesículas más pequeñas derivadas de la fusión exocítica de los cuerpos multivesiculares con la membrana de la bolsa del flagelo (Díaz Lozano et al., 2017).

Durante las primeras etapas de la infección, el contacto del parásito con las células del hospedador en el torrente sanguíneo promueve la liberación de las EVs capaces de inhibir el ataque del complemento (Díaz Lozano et al., 2017) y aumenta la infección en las células del hospedador (Retana et al., 2019). Así como alteran en el transcriptoma de las células, con las que interaccionan, la expresión de 322 genes de los cuales 168 se sobreexpresan y 154 se expresan a la baja, modificando tanto proteínas del citoesqueleto como proteínas apoptóticas o de señalización (Cornet-Gómez al., 2023). Pueden llegar a modular la

respuesta inflamatoria y, si esta función está desequilibrada, es una de las principales características responsables de la progresión de la EC. Por lo tanto, seguir estudiando estas EVs puede ser de gran interés para el descubrimiento de nuevas estrategias para el diagnóstico y tratamiento de la enfermedad (Cortes-Serra et al., 2022).

### 1.6.5 Formación de inmunocomplejos

En los pacientes con EC se ha estudiado la presencia de inmunocomplejos y su papel en algunas manifestaciones clínicas de la fase crónica (Chaves et al., 1982; Costa et al., 1991). Diversos trabajos consideraron estos inmunocomplejos como herramientas útiles para el diagnóstico (Petray et al., 1992; Ohyama et al., 2016), estudiando mediante análisis proteómico los antígenos del parásito presentes en los inmunocomplejos séricos de pacientes afectados por la enfermedad de Chagas crónica (ECC). En sus resultados encontraron la presencia de proteínas como las Transialidasas o GP63, proteínas propias de los tripanosomátidos (*T. cruzi*, *Leishmania* spp. o *T. brucei*). En el año 2017, se describió cómo los inmunocomplejos pueden estar formados por vesículas extracelulares del parásito e inmunoglobulinas del hospedador (Díaz et al., 2017). Estas EVs ligadas a inmunoglobulinas están presentes en el suero de pacientes con ECC (cardíacas, digestivas y asintomáticas) y podrían ser marcadores pronósticos de la patología de la enfermedad, actuando como portadores de una serie de proteínas específicas del parásito, como las proteínas pertenecientes a la familia MASP. Esta es una familia multigénica de aproximadamente 1.300 proteínas (dos Santos et al., 2012; de Pablos et al., 2016) que presentan una alta variabilidad, excepto en dos regiones de idéntica secuencia de nucleótidos. La región correspondiente a la secuencia C-terminal 5' (C-term) y la que codifica el péptido señal (SP), la región N-terminal 3'. El resto de la secuencia es

hipervariable, lo que hace que las proteínas MASP sean diferentes entre sí (de Pablos et al., 2016).

# 2. Justificación y objetivos de las publicaciones

## 2.1 Artículo 1: *Epidemiology and clinical description of Chagas disease in Valencia, Spain, from 2010 to 2020*

La enfermedad de Chagas (EC) es un importante problema de salud que afecta a más de 8 millones de personas en todo el mundo y es endémica en 21 países de América Latina. Los constantes flujos migratorios convierten a España en el primer país europeo en términos de carga de la enfermedad. Esta situación epidemiológica pone la necesidad de realizar acciones para logar un adecuado manejo diagnóstico y terapéutico de la enfermedad. En 2009 se creó, gracias a un grupo de trabajo multidisciplinar, un protocolo de actuación para ayudar a los profesionales sanitarios en la atención de las mujeres y los niños con riesgo de EC en la Comunidad Valenciana.

El **objetivo general** de este estudio es realizar un análisis exhaustivo de los pacientes diagnosticados con enfermedad de Chagas, tras la implementación del protocolo de cribado en la Comunidad Valenciana, con el fin de evaluar su impacto en la detección, seguimiento y manejo clínico de la enfermedad.

**Objetivos específicos:**

1. Determinar la prevalencia de la enfermedad de Chagas en el Hospital Universitario y Politécnico La Fe durante el período 2010-2020.
2. Evaluar el seguimiento de gestantes procedentes de zonas endémicas; así como el estado de salud de sus recién nacidos.
3. Analizar el uso del tratamiento para la EC, incluyendo una evaluación de los posibles efectos adversos.
4. Investigar las posibles coinfecciones con otras enfermedades infecciosas.

## 2.2 Artículo 2: *Circulating exovesicles in sera of chronic patients as a method for determining active parasitism in Chagas disease.*

Uno de los mayores retos en el manejo de la EC es la baja o nula parasitemia presente en la mayoría de los pacientes en fase crónica, complicando de esta manera el diagnóstico. En algunos pacientes inmunodeprimidos, tras el tratamiento, las formas quiescentes de *T. cruzi*, se reactivan provocando una parasitemia circulante elevada similar a la ocurrida en la fase aguda de la EC.

El **objetivo general** del estudio es investigar el uso de las EVs circulantes en suero como posibles marcadores de la presencia de formas metabólicamente activas del parásito en pacientes en la fase crónica de la enfermedad, y evaluar su relación con los inmunocomplejos formados por IgGs específicas, con el fin de mejorar el diagnóstico y seguimiento de la enfermedad.

**Objetivos específicos:**

1. Identificar nuevos marcadores útiles para el diagnóstico serológico de la enfermedad de Chagas crónica (ECC).
2. Evaluar la utilidad de las EVs circulantes en suero como método confirmatorio de la presencia de formas activas del parasito.
3. Investigar el papel de las EVs en la confirmación de la transmisión vertical en los recién nacidos.
4. Analizar la relación entre los diferentes isotipos de inmunoglobulinas IgG y los inmunocomplejos de las EVs en los pacientes con ECC.

## 2.3 Artículo 3: *Use of sera cell free DNA (cfDNA) and exovesicle-DNA for the molecular diagnosis of chronic Chagas disease*

Los métodos moleculares, como consecuencia de los fallos de extracción de los ácidos nucleicos o al mal manejo de las muestras de sangre, no alcanzan siempre la especificidad y sensibilidad requeridas para el diagnóstico de la enfermedad de Chagas crónica, ni para la detección del parásito en la sangre del recién nacido. La sensibilidad de estos métodos varía entre el 50 y el 90%. El **objetivo general** de este estudio es investigar la presencia de EVs procedentes del suero de los pacientes y el ADN circulante libre en suero, con el fin de optimizar el diagnóstico molecular de esta patología.

**Objetivos específicos:**

1. Evaluar el aumento de la sensibilidad en el diagnóstico de la enfermedad de Chagas mediante el uso de la PCR anidada o *nested*.
2. Investigar la presencia del ADN de las vesículas extracelulares circulantes en el suero o plasma de pacientes con EC, así como el ADN libre de células (cfDNA).
3. Comparar la sensibilidad y especificidad del ADN de las EVs circulantes en el suero de los pacientes o del cfDNA, con otros métodos moleculares de diagnóstico como la *nested* PCR para el ADNk o la qPCR empleando cebadores o *primers* para el ADN satélite presente en el núcleo del parásito.

# 3. Material y Métodos

## 3.1 Artículo 1: *Epidemiology and clinical description of Chagas disease in Valencia, Spain, from 2010 to 2020*

### 3.1.1 Diseño del estudio

Se realizó un estudio observacional, retrospectivo de todos los pacientes con enfermedad de Chagas en el Departamento de salud de Valencia La Fe entre enero de 2010 y diciembre de 2020. Se recogieron variables epidemiológicas, clínicas y terapéuticas de todos los casos de EC utilizando los programas del hospital de historia clínica, OrionClinic® y del laboratorio, Gestlab®. Comparamos los datos con la Red de Vigilancia Microbiológica de la Comunidad Valenciana (RedMIVA) y del Instituto Nacional de Estadística (INE).

### 3.1.2 Definición de los casos

El caso confirmado de EC se definió como un resultado positivo realizado por tres técnicas serológicas diferentes, utilizando como método de cribado una técnica de quimioluminiscencia en muestras de suero. También se realizó una PCR en sangre de los pacientes, que en los casos positivos sirvió para su confirmación, no descartándola así en los casos negativos cuando se trataba de un enfermo en fase crónica.

### 3.1.3 Variables recogidas

Se incluyeron diferentes variables:

- Edad, sexo, país y ciudad de nacimiento
- Sintomatología, tratamiento antiparasitario y dosis administrada
- Reacciones adversas al tratamiento y tratamiento de las reacciones adversas
- Embarazo y posible trasmisión vertical
- Trasplantes de órganos
- Familiares con EC
- Coinfección con VIH, *Strongyloides* y/o otros parásitos.

- Pruebas serológicas realizadas para detectar la EC
- Pruebas moleculares realizadas para detectar la EC
- Cifra de eosinófilos en sangre

### 3.1.4 Técnicas diagnósticas

En todo paciente procedente de área endémica, como técnica de cribado de EC, se realizó una serología por ELISA (Ortho Diagnostics, New Jersey) desde 2010 hasta 2013 y a partir de 2014 se sustituyó por el método de quimioluminiscencia de Liaison XL Murex (Diasorin, Italia) (Figura 7), usando antígenos recombinantes. Se realizó una inmunocromatografía, SD Chagas Ab rapid (Bioline, Korea) como segundo método confirmatorio (Figura 8), conforme lo estipulado por la Organización Mundial de la Salud (OMS) y se titularon los sueros mediante inmunofluorescencia indirecta por el método IFA Trinity® (Biotech, Irlanda) (Figura 9), resultando positivo por encima de la dilución 1:64.

El diagnóstico molecular se realizó mediante una *nested* PCR para detectar ADN del kinetoplasto de *T. cruzi.* La primera reacción se realizó con los *primers* 121 y 122 (que detectan ADN del kinetoplasto) mientras que para la segunda reacción se utilizaron los *primers* T3 y T4 para detectar una región de 150 pares de base, mejorando así la sensibilidad de la técnica (Figura 10).

Los pacientes con EC tuvieron un seguimiento en la Unidad de Enfermedades Infecciosas del centro para recibir un adecuado control y tratamiento de la enfermedad. Los estudios poblacionales se obtuvieron gracias al Instituto Nacional de Estadística (INE) y a la Red de Vigilancia Microbiológica de la Comunidad Valencia (RedMiva).

**Figura 7. Liaison® XL Murex (Diasorin).** **Figura 8. Inmunocromatografía SD Chagas (Bioline).**

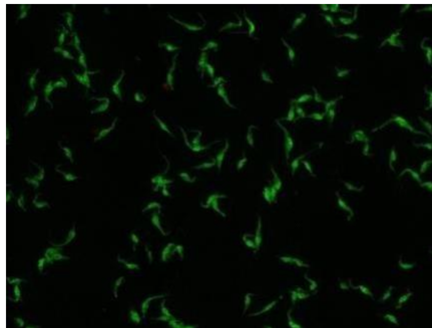


**Figura 9. IFI por método IFA Trinity® (Biotech).**

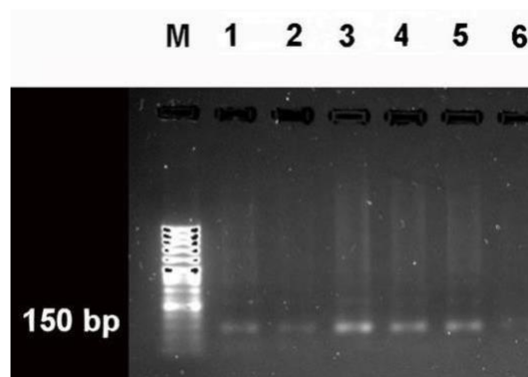



**Figura 10. Productos amplificados de 150 pb en un gel de agarosa.**

### 3.1.5 Sujetos del estudio

Los pacientes con EC fueron clasificados según la fase clínica en la que se encontraban y en función de la diferente sintomatología presentada:

- **Fase aguda:** en nuestro entorno es muy difícil ver a pacientes que se encuentran dentro de esta fase de la enfermedad, ya que, al encontrarnos en zona no endémica, no existe el vector transmisor del parásito. En este trabajo nos encontramos con recién nacidos, receptores sanos de donantes infectados y activaciones en los pacientes inmunosuprimidos que pueden presentar los síntomas propios de la EC aguda.
- **Fase crónica indeterminada:** la mayoría de los pacientes se presentan en esta fase. Son los que presentan EC durante más de 10 años y no presentan ningún tipo de sintomatología.
- **Fase crónica cardíaca:** Son los pacientes que presentan más de 10 años la EC y que durante el seguimiento realizado en la consulta de la Unidad de Enfermedades Infecciosas se observó alguna alteración en algunas de las pruebas realizadas (electrocardiograma, radiografía de tórax, ecocardiograma, resonancia cardíaca o holter).
- **Fase crónica digestiva:** Cumplen los mismos criterios que el grupo anterior, pero presentando diferentes alteraciones en el tubo digestivo. En este caso se realizó un enema opaco y un esofagograma para poder observar las anomalías digestivas.

### 3.1.6 Análisis estadístico

Las variables continuas fueron comparadas con T-student, mientras que en las variables categóricas se empleó la prueba de Pearson con IBM SPSS v12.0 y se consideró estadísticamente significativo un valor de $p<0,05$.

## 3.2 Artículo 2: *Circulating exovesicles in sera of chronic patients as a method for determining active parasitism in Chagas disease.*

### 3.2.1 Diseño del estudio

Se realizó un estudio observacional y retrospectivo en el Hospital Universitario y Politécnico La Fe (HUyP La Fe) en el periodo comprendido entre septiembre de 2017 y enero de 2020. Se seleccionaron al azar muestras de suero de pacientes con EC en el Servicio de Microbiología y se transportaron al laboratorio del Instituto de Biotecnología de la Universidad de Granada para realizar los ensayos de la investigación que dieron lugar a la publicación.

Se seleccionaron como controles negativos a pacientes procedentes de Bolivia que no tenían la enfermedad, de las mismas edades que nuestros casos y así poder calcular el valor de corte (valor de la media más tres veces el valor estándar de dicha media) para poder sustraer las absorbancias y poder representar los valores como valores relativos frente a cada uno de los casos, como se describe en el artículo. Los sujetos se fueron seleccionando al azar dentro de cada grupo de población. Por una parte, se seleccionaron recién nacidos de madres con EC junto con sus correspondientes madres. Por otra parte, los pacientes que tenían ECC se estratificaron en indeterminados, cardiacos y digestivos, así como un grupo minoritario que poseían estas dos últimas afectaciones.

Se estudiaron poblaciones de dos áreas geográficas diferentes. La primera población fueron pacientes bolivianos residentes en Valencia, mientras que la segunda, y a modo de control y como prueba de concepto, una población rural de pacientes de Panamá, a la que se le habían realizado tres pruebas serológicas diferentes (prueba rápida, ELISA comercial y un *Western blot*) cuando no existían concordancias de resultados, a fin de poder comparar la especificidad que mostraba el ensayo con los inmunocomplejos y EVs circulantes. Se incluyeron como control para esta prueba algunos sueros con anticuerpos de

leishmaniosis y sífilis, que puedan dar reacciones cruzadas en pruebas inmunológicas, como se describe en el artículo publicado. En el primer caso contamos con 63 adultos, 16 recién nacidos, analizados al mes y nueve meses del nacimiento y sus madres junto a 13 controles En el segundo caso, de los 106 sueros analizados, 53 resultaron positivos. Todos los sueros positivos mostraron PCR positiva usando las sondas 121/122 para ADN del Kinetoplasto. Se usó 1 ml de sangre periférica tratada con guanidina (6 M). La extracción de los ácidos nucleicos se realizó manualmente con el procedimiento de Fenol/Cloroformo/ alcohol Isoamílico monitorizándose espectrofotométricamente.

### 3.2.2 Variables recogidas

Se incluyeron variables demográficas (edad, sexo y país de nacimiento), clínicas (tratamiento antiparasitario y sintomatología) y analíticas (pruebas serológicas y moleculares)

### 3.2.3 Técnicas de aislamiento, detección y cuantificación de vesículas extracelulares

Se utilizaron dos métodos para conseguir el **aislamiento** de las vesículas extracelulares:

Ultracentrifugación (*Gold Standard*)

La purificación y aislamiento de los inmunocomplejos circulantes (Ig-EVs) se llevó a cabo utilizando la metodología descrita previamente por nuestro grupo (Díaz et al., 2017; Retana et al., 2019) mediante un procedimiento mixto de centrifugaciones a 1.500 x g y a 17.000 x g y con filtración a través de filtros de 0,45/0,22 µm, con el objeto de eliminar cuerpos apoptóticos y ectosomas. A continuación, se realizó una ultracentrifugación a 110.000 x g durante un mínimo de 2 horas en tubos de microcentrífuga (Hitachi No 1508) a 4ºC en una centrífuga CP100NX (Hitachi Koki, Tokyo, Japan) con ángulo de rotor fijo P70A.

Filtración mediante concentradores

Como método alternativo para la concentración de las EVs y a fin de los facilitar el uso a centros de diagnóstico con limitadas capacidades instrumentales, se puso a prueba la

purificación de dichos inmunocomplejos presentes en el suero de los pacientes, ya que las técnicas de ultracentrifugación pueden no ser asequibles en los laboratorios de la mayoría de los hospitales. El método que utilizamos con estos concentradores de proteínas fue descrito previamente por Orrego et al. (2021) y Ramírez et al. (2018) con algunas modificaciones.

Para ello, se diluyó el suero de cada paciente (1 mL) con 5 mL de PBS ultrafiltrado por 0,22 µm. Tras lo que se realizó una centrifugación a 1.500 x g durante 10 minutos y el sobrenadante resultante se filtró a través de un tamaño de poro de 0,45 µm. El sobrenadante se sometió a un paso adicional de una centrifugación a 3.500 x g durante 20 minutos. Posteriormente el sobrenadante final diluido en PBS se aplicó al concentrador de proteínas Vivaspin® (Sartorius Lab Instrumen, Goettingen, Germany) (Figura 11) hasta conseguir un tamaño de 100K (100,000 MWCO), centrifugándose nuevamente a 6.000 x g durante 1 hora a 4ºC.

**Figura 11. Concentradores de proteínas Vivaspin® (Sartorius).**

La **evaluación de la pureza y la detección** de las EVs purificadas se realizó mediante un equipo Nanoparticle Tracking Analysis (NTA) (NanoSight Ltd., Amesbury UK) y mediante Microscopía Electrónica de Transmisión (MET).

Nanoparticle Tracking Analysis (NTA)

Este equipo sigue la trayectoria de todas las nanopartículas presentes en la muestra durante un tiempo determinado, basándose en el movimiento browniano. El equipo es capaz de extrapolar el diámetro hidrodinámico de todas ellas, dando un resultado de la distribución de tamaños (Dragovic et al., 2011). Una de las ventajas de esta técnica es que aporta una gran información sobre un importante número de nanopartículas, por lo que el poder estadístico es muy robusto. Sin embargo, la desventaja es que no genera imágenes que proporcionen información sobre la forma de las partículas (Théry el at., 2018).

Para calcular las medidas de las partículas se realizaron sucesivas diluciones hasta encontrar la concentración óptima que permitiera un correcto enfoque de la muestra con un número suficiente de trayectorias seguidas (con más de 500 trayectorias se puede considerar significativo). Se utilizó un láser de 405 nm, con una cámara complementaria metal-óxido semiconductora y se tomaron y analizaron tres vídeos de 60 segundos. El análisis se llevó a cabo con NTA 2.3 image-analysis software (NanoSight Ltd., Amesbury UK).

Microscopía Electrónica de Transmisión (MET)

Para poder visualizar y evaluar la pureza de las EVs purificadas se llevó a cabo su observación utilizando microscopía electrónica de trasmisión.

Las muestras obtenidas del último paso se resuspendieron en 30 µL de Tris-HCl (pH 7,3), y se aplicaron directamente 5 µL de la suspensión sobre rejillas de observación de microscopia electrónica. La rejilla de níquel cubierta con una película de carbón se dispuso sobre la gota de la suspensión de las EVs que se incubó durante 5 minutos con objeto de favorecer la adsorción de las EVs a la rejilla. Posteriormente la rejilla fue lavada con PBS filtrado por 0,1

μm durante 1 minuto. La preparación se contrastó colocando la rejilla sobre una gota de acetato de uranilo al 1% en solución acuosa durante 1 minuto y se secó sobre un papel de filtro. Las EVs se fijaron con vapores de glutaraldehído, incubándose durante una hora a temperatura ambiente. La observación de la muestra se realizó utilizando el microscopio electrónico LIBRA 120 PLUS (Zeiss, Jena, Alemania) en la unidad de microscopía electrónica del Centro de instrumentación Científica, en la Universidad de Granada. El tamaño de las nanopartículas se midió utilizando la escala de medición del microscopio y el software Image J 1.41.

Asimismo, se confirmó la presencia de las EVs por Microscopia de Fuerza Atómica siguiendo la metodología previamente descrita por Prescilla-Ledezma et al (2022).

Para estos experimentos, se depositaron 8 μL de la suspensión sobre una lámina de mica moscovita que sirvió como sustrato. Las muestras se incubaron durante 15 minutos. Posteriormente, se realizaron 3 lavados con agua ultrapura MilliQ® (Millipore, Burlington, MA, USA), y las muestras se secaron bajo un flujo suave de argón antes de la visualización. El análisis de microscopía de fuerza atómica se llevó a cabo utilizando el equipo NX-20® (Park Systems, Suwon, Corea). Las imágenes se adquirieron y procesaron como lo describen Retana et al. (2019) utilizando el software XEI® (Park Systems, Suwon, Corea).

Para la **cuantificación** de proteínas se utilizó el método *MicroBCA™ Protein assay kit* (Thermo Fischer Scientific, Waltham, MA, EEUU). El uso de este kit permite la detección colorimétrica de las proteínas. Con el fin de reducir el uso de reactivos y de la muestra, seguimos el protocolo del fabricante con algunas modificaciones. Se preparó una curva patrón con las siguientes concentraciones de albúmina: 0-0,01-0,02-0,1 y 0,2 μg/μl, diluidos en una mezcla de disoluciones comerciales A, B y C en una proporción 25:24:1 respectivamente. De la misma manera, la muestra se diluyó en esta mezcla, se incubó a 60ºC

durante 30 minutos, y finalmente se realizó la medida de absorbancia a 562 nm en un espectrofotómetro de placas. Los valores de la curva patrón seguían una recta de regresión y una vez calculada el valor de la r, los valores de absorbancia de las muestras problema fueron extrapolados, conociéndose así las concentraciones proteicas de las muestras del experimento.

### 3.2.4 Técnicas ELISA "Enzyme-Linked ImmunoSorbent Assay"; Ensayo por inmunoadsorción ligado a enzimas

**Detección de Anti-MASP y Anti-*T. cruzi* de EVs extraídas de sueros de pacientes con EC.**

Se sensibilizó la placa de ELISA (Thermo Fisher Scientific) con un volumen de 100 µL por pocillo y una concentración de 5 µg/µL de proteína procedente de las EVs, de los sueros de los pacientes, diluido en tampón carbonato a pH 9,6. La placa se incubó en agitación durante 8 horas a 4ºC. Posteriormente, se realizaron 2 lavados con la solución de lavado (PBS-0,1% Tween 20) y se procedió al bloqueo de los pocillos para evitar uniones inespecíficas del anticuerpo. Se agregaron 250 µL de solución de bloqueo (solución de lavado + 5% de leche en polvo) y se incubó toda la noche a 4ºC. Después de los dos siguientes lavados con PBST, se añadieron 100 µL de suero primario de *T. cruzi* (1:2000) o anti-MASP SP (1:6400) de rata diluidos en PBS a cada pocillo, incubándose durante 2 horas en agitación a temperatura ambiente. Después de esta sensibilización, las placas se lavaron 3 veces con PBST, al que se agregaron 100 µL de IgG policlonal de cabra anti-rata marcado con peroxidasa (Sigma- Aldrich, ref: A9037) a una dilución de 1:1000 en PBS. La mezcla se incubó a temperatura ambiente en agitación durante 1 hora.

Después de la incubación con los anticuerpos secundarios, las placas se lavaron cuatro veces tras lo que se añadió O-fenildiaminobencidina más $H_2 0_2$ al 30% (Sigma-Aldrich) a 0,005 M de tampón fosfato-citrato, pH 5,0 como sustrato de peroxidasa. Las placas se incubaron 15

minutos a 27ºC y la reacción se detuvo con una solución de $H_2S0_4$ 0,1M. La lectura de la absorbancia se llevó a cabo en un lector ELISA Multiskan Spectrum (Thermo Fisger Scientific) a 492 µm.

**Detección de isotipos de IgG en los inmunocomplejos formados en los pacientes con ECC.**

Para la realización de esta técnica, previamente se separaron las IgG de los inmunocomplejos mediante tratamiento con tampón glicina-HCL 0,1 M a pH 4, ultracentrifugándose a fin de separar el sobrenadante con las IgGs de las EVs. Las IgGs se purificaron mediante columnas Protein G HP SpinTrap/Ab Spin Trap™ (GE Healthcare Life Sciences, 28-9031-34) siguiendo las instrucciones del fabricante.

La tipificación de los diferentes isotipos de IgG, así como de subclases de inmunoglobulinas, se realizó mediante ELISA siguiendo la metodología descrita anteriormente. Los anticuerpos primarios fueron a los diferentes isotipos de IgG anti-humano obtenidos en rata o ratón en una dilución 1:1000. Se incubaron durante 1 hora en agitación a temperatura ambiente. Se emplearon anticuerpos secundarios marcadas con HRP peroxidasa (excepto para el caso de la IgG4 donde el anti IgG4 ya viene marcada) Los anticuerpos secundarios se añadieron en una dilución 1:1000 de anti-rata (Sigma-Aldrich, ref: A9037) o anti-ratón (Dako, ref: P0447) incubándose durante una hora a temperatura ambiente.

Se procedió al lavado de la placa con PBST a la que se añadió 100 µl de peroxidasa como sustrato de la reacción, incubándose 20 minutos en agitación a temperatura ambiente en oscuridad. Finalmente, la reacción se detuvo con 50 µl de HCL 3M y la lectura se realizó a 492 nm en el espectrofotómetro (Thermo Fisher Scientific).

### 3.2.5 Análisis estadístico

Se usó la prueba de Shapiro para comprobar la normalidad de la distribución de los datos. Los datos distribuidos normalmente se compararon con la prueba ANOVA y Turkey y los

no paramétricos se compararon con la U de Mann-Whitney y la de Kruskal-Wallis. Los datos relacionados no paramétricos se estudiaron con la prueba de rangos con signos de Wilcoxon al que se aplicó el método del nivel de significancia corregido por Bonferroni. Los análisis estadísticos se realizaron con R y se consideró significativo para una $p<0,05$.

## 3.3 Artículo 3: *Use of sera cell free DNA (cfDNA) and exovesicle-DNA for the molecular diagnosis of chronic Chagas disease*

### 3.3.1 Diseño del estudio

Para este artículo se utilizaron tanto muestras de suero como de plasma recibidas en el Servicio de Microbiología del Hospital Universitario y Politécnico La Fe durante 2011 a 2020, de pacientes con la enfermedad de Chagas.

Para la detección del ADN de las EVs en suero se seleccionaron al azar 50 muestras con serología positiva, 25 *nested PCR* positivas (121F/122 R) y posterior amplificación con las sondas (T3 F/T4 R) y 25 *nested* PCR negativas. Sin embargo, para la detección de ADN libre en suero se realizó un pool de las muestras de diferentes pacientes, agrupados según el tipo de sintomatología que presentaban estos pacientes.

### 3.3.2 PCR anidada o *nested*

Se extrajeron 5 mL de sangre en tubos de vacutainer con EDTA y se realizó un pretratamiento con buffer de lisis 1:1 (guanidina 6M, 10 mM urea, 10 mM TRIS-HCL, 20% (v/v) Triton X-100; pH 4,4), incubándose a 70ºC 10 minutos y conservándolas 48 horas a temperatura ambiente antes de proceder al proceso de purificación de ADN.

Se realizó la extracción del ADN por el kit de Maxwell® Blood DNA Purification (Promega Biotech) según las indicaciones del fabricante.

Se amplificó el ADN por una *nested*, PCR para la detección del ADN del kinetoplasto se emplearon los *primers* 121F (5´-AAATAATGTACCGGKGAGATGCATGA-3`) y 122 R (5´GGTTCGATTGGGGTTGGTGTA ATATA-3´) con el que se obtuvo en los casos positivos un amplicón de 330 pb, con este producto amplificado se realizó una segunda amplificación con los *primers* T3 F (5´- TC TTT GGT GTG ATC GTT AC-3´) y T4 R (5´-TAC ATT CTA TTT CTT CTC TG-3´) para obtener un producto de 150 pb y se procedió a

la visualización mediante electroforesis en un gel de agarosa 2%.

### 3.3.3 ADN de exovesículas y ADN libre de células (cfDNA) en suero

El aislamiento y purificación de las EVs en suero ya ha sido comentado anteriormente, siguiendo los métodos descritos por Díaz et al (2017) y Retana et al (2019). El aislamiento del ADN en las vesículas fue realizado gracias al protocolo descrito por Orrego et al (2021). El sedimento que contenía EVs del suero se trató con 2 unidades de ADNasa I en buffer TAE (Tris-acetato 0,04M, EDTA 1mM, pH 8,0) a 37ºC durante 30 minutos para eliminar el ADN adherido externamente a las EVs. Después del tratamiento, la enzima se inactivó con calor a 70ºC en una solución con EDTA 50 mM. Después de eliminar los ácidos nucleicos libres de la suspensión de EVs, se lisaron 100 µl de EVs con 200 µl de tampón de lisis (Tris-HCL 30 mM, pH 8,0, EDTA 10mM, SDS al 1%) suplementado con 20 µl de proteinasa K (0,1 mg/mL), y se incubó a 56ºC durante 1 h. Finalmente, la purificación y precipitación de los ácidos nucleicos se realizaron siguiendo el método tradicional de fenol-cloroformo mezclando 320 µL de suspensión de EVs lisados con 320 µL de reactivo de fenol-cloroformo-Alcohol isoamílico 25:24:1 (v/v( (Sigma). La mezcla se agitó y se centrifugó a 14000 x g durante 10 minutos. Luego se recogió la fase acuosa y el ADN se precipitó con 1/10 vol de 3M, pH 5 y 2,5 vol de etanol absoluto de calidad HPLC. La mezcla se incubó durante la noche a -20ºC y se centrifugó a 15000 x g durante 10 minutos a 4ºC. Se descartó el sobrenadante y el precipitado se lavó dos veces con etanol frío al 70%. Finalmente, el sedimento se secó en un equipo Jouan Thermo Speed Vac™ (RC1010) a 20ºC y si diluyó en 20 µL de agua Milli-Q®

Por otra parte, para el aislamiento del ADN libre circulante del parásito, a partir del suero de los pacientes, se utilizó el kit de MagMAX$^{TM}$ (Thermo Fisher Scientific), siguiendo las instrucciones del fabricante. Primero, los pools de sueros de 100 µl se centrifugaron a 1600

x g durante 10 minutos a 4ºC. Una vez finalizada la primera centrifugación, el sobrenadante se sometió a una segunda centrifugación a la misma velocidad y durante el mismo tiempo que la anterior para eliminar posibles restos celulares presentes en el suero. A continuación, las proteínas que se encontraban en el sobrenadante se digirieron durante 20 minutos a 60ºC con 2 µL de Proteinasa K (20 µL/mL). Al producto de la digestión, se le añadieron 150 µl de tampón (Tris-HCL 30M, pH 8,0, EDTA 10 mM, SDS al 1%) junto con 5 µl de solución de perlas magnéticas. El volumen resultante (255 µL) de la mezcla se agitó durante 10 minutos y luego se centrifugó a 14.000 x g durante 10 segundos. El sedimento resultante se lavó dos veces, primero con 500 µL de tampón de lavado del kit y luego con 500 µL de etanol al 80%, y se sometió a centrifugación (20 segundos a 13.000 x g). Finalmente, el sedimento se secó y se resuspendió en 50 Ml de agua Milli-Q®. Se comprobó la presencia de las EVs y se procedió a la amplificación del ADN utilizando los *primers* correspondientes. El producto final se visualizó por electroforesis en un gel de agarosa al 2%.

### 3.3.4 PCR a tiempo real (qPCR)

Para conocer si es posible cuantificar el ADN presente en las EVs, se amplificó la secuencia satélite mediante PCR a tiempo real cuantitativa (qPCR), siguiendo el protocolo descrito por Ramírez et al. (2015) con algunas modificaciones. La curva estándar se generó usando una cantidad inicial de 1,7 ng/µL procedente del ADN del tripomastigote, determinándose el límite de detección de ADN que resulto de 1,7 fg, con una eficiencia del 101% y un R2 de 0,99. Las muestras y la curva estándar se amplificaron de acuerdo con las instrucciones del fabricante para SsoAdvanced™ Universal Probes Supermix (Biorad 172-5281), utilizando 700 nM de concentración de primeres y 200 nM de sonda. La cuantificación del ADN se midió en un termociclador Bio-Rad CFX96.

Todos los *primers* utilizados en este estudio se resumen en la Tabla 1.

**Tabla 1.** ***Primers*** **utilizados para la realización de las PCR.**

| *Nombre* | Secuencia 5`→3` | Tm (ºC) | Tipo | Tamaño (bp) | Gen |
|---|---|---|---|---|---|
| *SATF*<br>*SATR* | GCAGTCGGCKATCKTTTTCG | 60,1 | PCR | 120 | ADN satélite (nuclear) |
| | TTCAGRGRRGRRRGGRGRCCAGTG | 58,5 | PCR | | |
| *121F*<br>*122R* | AAATAATGTACCGGKGAGATGCATGA | 65 | PCR | 330 | ADN kinetoplasto |
| | GGTTCGATTGGGGTTGGTGTAATATA | 66 | PCR | | |
| *T3*<br>*T4* | TC TTT GGT GTG ATC GTT AC | 58 | *Nested* | 150 | ADN kinetoplasto |
| | TAC ATT CTA TTT CTT CTC TG | 52 | *Nested* | | |
| *Cruzi1*<br>*Cruzi2*<br>*Cruzi3* | ASTCGGCTGATCGTTTTCGA | 56,6 | *Real time qPCR* | | ADN satélite (nuclear) |
| | AATTCCTCCAAGCAGCGGATA | 56,4 | *Real Time qPCR* | | |
| | FAM-CACACACTGGACACCAA-NFQ- MGB | 52,2 | *Real Time qPCR-* Sonda | | |

# 4. Principales resultados

### 4.1 Artículo 1: *Epidemiology and clinical description of Chagas disease in Valencia, Spain, from 2010 to 2020*

- En los 11 años del estudio se realizaron 3.713 serologías para la detección de EC, 3.159 mujeres y 554 hombres, con un 11,7% de prevalencia, siendo el 70% mujeres. La población pediátrica tuvo una prevalencia del 24,5%, mientras que la de los adultos fue de un 75,5%. Hubo discrepancias en 14 muestras, considerándolas falsos positivos.
- La PCR detectó ADN del parásito en un 20% de los casos positivos.
- Bolivia fue el país con más representación, encontrando una prevalencia del 91,2%, seguido de Ecuador (1,8%), Argentina (1,5%), Colombia (0,9%), Brasil (0,3%), El Salvador (0,3%) y Venezuela (0,3%). Dentro del grupo de bolivianos, el departamento de origen más frecuente fue Santa Cruz en el 38, 2% de los pacientes. Todos los niños estudiados nacieron en España, con excepción de 2 que habían nacido en Bolivia.
- Se realizó un seguimiento a 106 niños y niñas nacidos de madres con EC y en 7 (6,6%) se detectó la enfermedad, aunque solamente se pudo confirmar que hubo transmisión vertical en 4 casos (3,8%).
- Se implementó el tratamiento con benznidazol en 204 pacientes, 5 niños y 199 adultos, de los cuales un 54% desarrollaron reacciones adversas (RA). La RA más frecuentemente encontrada fue la aparición de lesiones dermatológicas.
- Solamente la población adulta presentó algún tipo de sintomatología asociada a la EC. De los pacientes estudiados, un 26,6% tuvieron alguna afectación, siendo más frecuentes en hombres que en mujeres (44,4% *vs.* 20,1 %).

- Cuando se estudiaron las coinfecciones con otros parásitos, de las 84 muestras analizadas para *S. stercoralis,* se detectó un 12% de prevalencia y casi un 20% de los estudiados para otro tipo de parásitos intestinales.
- Durante este periodo, un receptor de trasplante hepático sano se contagió por un donante con serología positiva, detectándose mediante PCR a los 43 días post trasplante.

### 4.2 Artículo2: *Circulating exovesicles in sera of chronic patients as a method for determining active parasitism in Chagas disease.*

- Respecto a los resultados obtenidos en el estudio comparativo de los métodos de aislamiento de EVs, tanto usando la técnica de ultracentrifugación como la de filtración por los concentradores de proteínas, observamos que el tamaño encontrado mediante NTA fue muy similar por ambos, no encontrando diferencias significativas. Sin embargo, usando la ultracentrifugación, obtuvimos mayor concentración de proteínas (27,9±10,8 µg/µl *vs*. 9,5±8µg/µl) ($T(46) = 6,7$ $P < 0.00001$).
- Usando antígenos para reconocer las EVs purificadas, observamos que los valores de absorbancia más elevados los encontramos con el método de ultracentrifugación.
- Cuando estudiamos los valores de absorbancia comparando ambos marcadores, observamos que la absorbancia fue mayor utilizando anti-*T. cruzi* que anti-MASP SP. Las EVs reaccionaron frente a anti-*T. cruzi* en 23 de 24 pacientes con sintomatología indeterminada, mientas que cuando usamos suero anti-MASP SP, 20 de las 24 presentaron una absorbancia mayor al punto de corte. En los 39 pacientes sintomáticos, 35 fueron detectados frente a anti-*T. cruzi* y 32 cuando usamos suero anti-MASP SP.

- La absorbancia media usando anti-*T. cruzi* en los pacientes cardiacos, fue de 1,21, mientras que en los gastrointestinales fue de 1,19 y en los que presentaron ambas afectaciones fue de 1,31.
- Por otra parte, utilizando suero anti-MASP SP, la absorbancia media en los pacientes cardiacos fue de 0,72, en los gastrointestinales de 0,66 y en ambas afecciones de 0,21.
- Se detectó la presencia de las EVs en las pacientes embarazadas. El seguimiento realizado a sus recién nacidos, al mes y a los 9 meses de vida, mostró la presencia de las EVs y el descenso de estas a medida que avanzaba el tiempo. Fueron enfrentados a anti-*T. cruzi* observamos una absorbancia media de 2,08 (95% CI: 1,77-2,27) en el primer mes de nacimiento, descendiendo a 1,24 (95% CI: 0,74-1,61) a los 9 meses. Además, se encontró una correlación de Spearman entre los valores de absorbancia al mes y a los 9 meses (Rho 0,72; valor p = 0,009). En las muestras correspondientes a los niños 5 y 13, los valores de absorbancia fueron iguales o inferiores al valor de corte utilizando el inmunosuero contra el antígeno total de *T. cruzi* a los 9 meses. La absorbancia de la muestra correspondiente al paciente 16, un niño que recibió tratamiento (benznidazol 10mg/kg durante 10 días) a los dos meses de nacer debido a la PCR positiva de la madre durante el embarazo y posteriormente del niño al nacer, disminuyó a los 9 meses de forma considerable respecto a la que presentó al mes de nacimiento.
- Por último, después de la purificación de los inmunocomplejos circulantes (IgGs-EVs) del suero de los pacientes con EC, se realizó un estudio de los diferentes isotipos de IgGs observando que la IgG2 y la IgG4 presentaron niveles estadísticamente más elevados.

### 4.3 Artículo 3: Use of sera cell free DNA (cfDNA) and exovesicle-DNA for the molecular diagnosis of chronic Chagas disease

- Este trabajo confirmó la presencia de las EVs aisladas por microscopía electrónica y NTA. El tamaño medio de las partículas fue de 119,92 ±18,41 nm, medidas por microscopía electrónica (figura 12.A). Mediante NTA se observaron diferentes poblaciones de EVs, encontrando un pico de mayor densidad en 163 nm y una segunda población de 231 nm (figura 12.B), quizás correspondiente a agregados de las partículas.

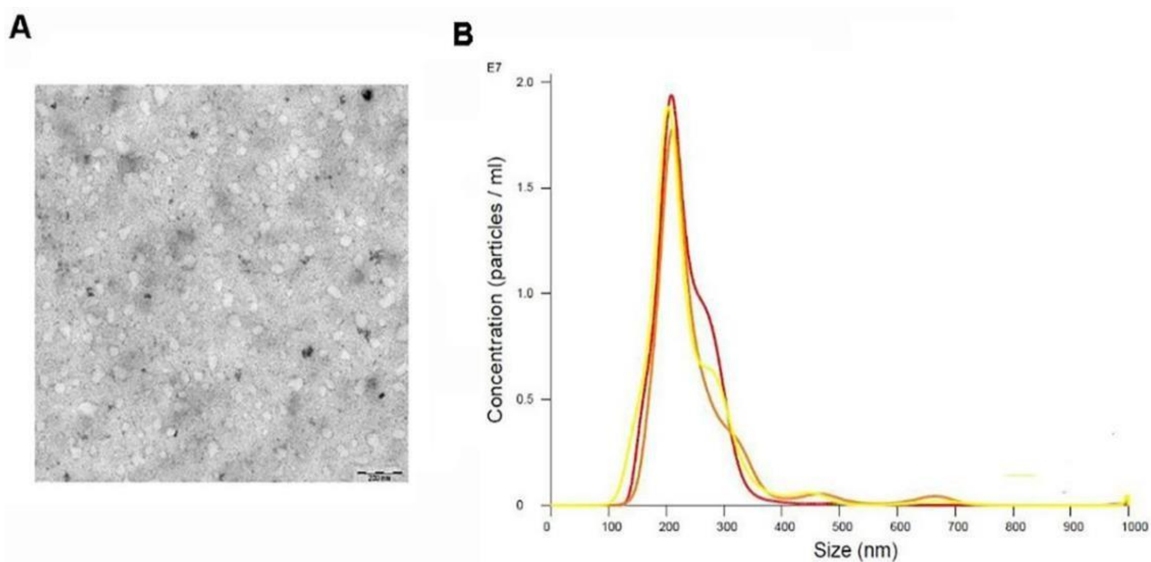



Figura 12 A. Microscopía B. NTA.

- Se seleccionaron al azar 50 pacientes, 25 muestras de pacientes con EC que presentaban *nested* PCR positiva y 25 con PCR negativa. A las 50 muestras se les realizó el aislamiento de ADN de EVs y de ADN libre circulante.

- <u>Análisis PCR usando ADN de las EVs</u>: 25 muestras resultaron positivas usando los *primers* 121F-122R (figura 13 A) y 24 fueron positivas utilizando los *primers* SAT (figura 13 B).

- <u>Análisis PCR usando ADN libre en suero:</u> en esta ocasión los 6 pool de muestras correspondientes a los pacientes con clínica cardíaca, digestiva e indeterminada fueron positivos utilizando los *primers* SAT (figura 13 C).

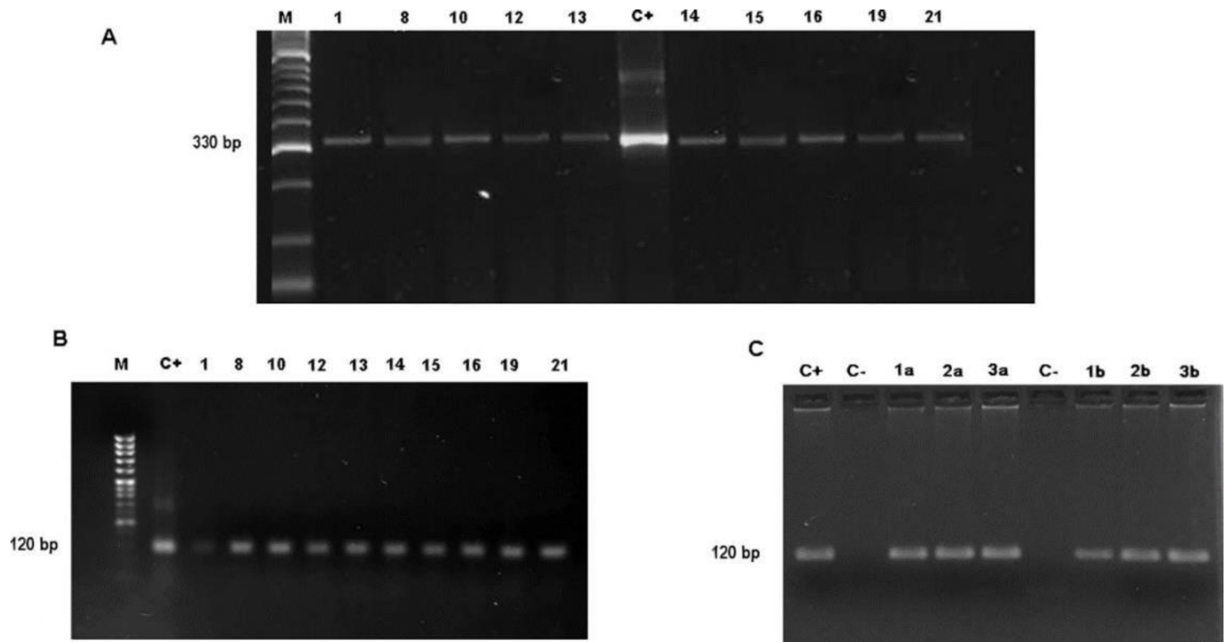



Figura 13. A. *Primers* 121F-122R B. *Primers* SAT
C. Pool cardiacos/digestivos/indeterminados
*primers* SAT.

- Para determinar el límite de detección y el límite de cuantificación para detectar el ADN de EVs, se estudió en 6 muestras, siguiendo el método descrito por Ramírez et al., 2015 analizándose mediante qPCR. El resultado de la cuantificación fue de 1,9 fg y 2,35 fg, respectivamente. Las muestras analizadas fueron positivas, si bien no se interpolaron en la curva estándar.

# SECCIÓN II: TRABAJOS PUBLICADOS

# *Epidemiology and clinical description of Chagas disease in Valencia, Spain, from 2010 to 2020.*

Noelia Lozano, Carmen Lloret-Sos, María José Giménez-Martí, José Miguel Sahuquillo-Arce, María Dolores Gómez-Ruiz, María Trelis, José Luis López-Hontangas, Antonio Osuna, Eva Calabuig.

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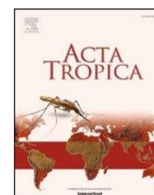

# Epidemiology and clinical description of Chagas disease in Valencia, Spain, from 2010 to 2020

Noelia Lozano [a,*], Carmen Lloret-Sos [b], María José Giménez-Martí [b], José Miguel Sahuquillo-Arce [c,d], María Dolores Gómez-Ruiz [b], María Trelis [e], José Luis López-Hontangas [b], Antonio Osuna [f], Eva Calabuig [g]

[a] Area of Parasitology, Department of Pharmacy and Pharmaceutical Technology and Parasitology, University of Valencia, Valencia, Spain and Servicio de Microbiología y Parasitología Clínica, Hospital Universitario y Politécnico La Fe, Valencia, Spain

[b] Servicio de Microbiología y Parasitología Clínica, Hospital Universitario y Politécnico La Fe, Valencia, Spain

[c] Respiratory Infections Research Group, Valencia-Health Research Institute La Fe, Valencia, Spain

[d] Servicio de Microbiología, Hospital Virgen de La Peña, Puerto del Rosario, Spain

[e] Area of Parasitology, Department of Pharmacy and Pharmaceutical Technology and Parasitology, University of Valencia, Valencia, Spain and Joint Research Unit on Endocrinology, Nutrition and Clinical Dietetics, University of Valencia-Health Research Institute La Fe, Valencia, Spain

[f] Grupo de Bioquímica y Parasitología Molecular (CTS 183), Departamento de Parasitología, Campus de Fuentenueva, Instituto de Biotecnología, Universidad de Granada, Granada, Spain

[g] Unidad de Enfermedades Infecciosas, Servicio de Medicina Interna, Hospital Universitario y Politécnico La Fe, University of Valencia-Health Research Institute La Fe, Valencia, Spain

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ABSTRACT

Chagas disease (CD), caused by the flagellate protozoan parasite *Trypanosoma cruzi*, is endemic in 21 American countries. It is estimated that about 6–8 million people are infected. Nevertheless, CD should be now considered a worldwide-distributed disease due to migratory movements from endemic to non-endemic regions where the infection risk is related to transplacental transmission to newborns from infected mothers or through blood or organ donations from infected individuals. Spain is the non-endemic country with the highest burden of CD in Europe. This study aims to assess the prevalence and perform a descriptive analysis of patients with CD at Hospital Universitari i Politécnic (HUiP) in Valencia, Spain, from 2010 to 2020, to raise awareness about the importance of the disease.We compared data collected from official sources (National Institute of Statistics and Microbiological Surveillance Network of the Comunitat Valenciana) as well as data from the Health Department-Valencia La Fe, Valencia (Spain). A total of 3713 subjects were analyzed, 3159 women and 554 men, of which 433 were positive, prevalence of 11.7 %. Pediatric population accounted for 106 (24.5 %) while adult population for 327 (75.5 %). Most migrants were from Bolivia (298, 91.1 %). Regarding women, 200 (64 %) were of childbearing age and 90 (45 %) of these were pregnant. DNA detection by PCR was positive only in 4 newborns (3.8 %). Treatment with benznidazole was implemented in 204 patients and 107 developed adverse drug reactions (ADRs). In conclusion, the prevalence of CD in our health department is not negligible and poses a challenge for the health system; worrisomely, many patients remain undiagnosed and untreated.

## 1. Introduction

Chagas disease (CD) is a major health problem caused by *Trypanosoma cruzi*, affecting approximately 6–8 million people worldwide and being endemic in 21 Latin American countries. However, due to constant population flows, Spain now bears the highest burden of CD in Europe, with an estimated 55,000 cases (Basile et al., 2011; Sulleiro et al., 2021). This epidemiological situation necessitates adequate diagnostic and therapeutic management. Thus in 2009, a multidisciplinary group of experts created the CD protocol for the care and attention of women and children at risk in the Comunitat Valenciana, Spain (Bayón et al., 2009). Since then, more regional governments have joined this initiative (González-Tomé et al., 2013).

In endemic areas, the main transmission mechanism is through

* Corresponding author at: Servicio de Microbiología, Hospital Universitari i Politècnic La Fe, Avenida Fernando Abril Martorell, 106, 46026 Valencia, Spain.
*E-mail address:* noloro2@alumni.uv.es (N. Lozano).

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contact with triatomine feces (vector route), but outbreaks can also be caused by consuming food or beverages contaminated with infected triatomines. Alternative transmission mechanisms include maternal-fetal transmission, organ donations, blood transfusions, and laboratory accidents. Thus, in countries without vector transmission, disease control focuses on preventing both vertical transmission and transmission via organ or blood donation (P´erez-Molina and Molina, 2018).

The disease course is characterized by an acute phase followed by a chronic phase, which is symptomatic in most cases. Up to 40 % of cases present complications, whether cardiac, digestive, or both, with dilated cardiomyopathy (DCM) being the most serious manifestation (Marin-Neto and Rassi, 2009; Roca Saumell et al., 2015).

In Spain, the treatment of choice is benznidazole (BNZ), although its effectiveness varies depending on the disease phase and it is contraindicated during pregnancy. In congenital infections, early detection and treatment achieve cure rates of 100 % (Marin-Neto and Rassi, 2009). However, BNZ is associated with a high rate of adverse drug reactions (ADRs), mainly cutaneous (Kratz, 2019), which increase with the patient's age, resulting in low adherence, especially in adults (Pereiro et al., 2018).

The objective of our research was to perform a descriptive analysis of patients with CD at the Hospital Universitari i Polit´ecnic (HUiP) La Fe in Valencia, Spain, to raise awareness about the real burden of CD, and to highlight the importance of accurate diagnosis and follow-up for each patient, thus preventing new cases and avoiding the long-lasting lethal effects.

## 2. Materials and methods

The Health Department-Valencia La Fe, Valencia (Spain) is responsible for the healthcare of 210,000 citizens, which includes HUiP La Fe, a Chronic Hospitalization Unit, a Specialty Centre and 6 Primary Care Centres. HUiP La Fe is a 1000-bed hospital providing tertiary care for the management of CD in the metropolitan area of Valencia. From January 2010 to December 2020, we conducted a retrospective and observational study on migrants from CD-endemic areas, including 3713 subjects (3159 women and 554 men), whose sera were analyzed in the Microbiology and Parasitology Laboratory.

Screening to detect CD was carried out on all patients' sera according to World Health Organization (WHO) recommendations. Two serological tests with different methods were used on each patient: an enzyme-linked immunosorbent assay (Ortho Diagnostics, New Jersey) until 2013, or LiaisonXLmurex chemiluminescence kit (Diasorin, Italy) from 2014 to 2020, and SD Chagas Ab Rapid immunochromatography (Bioline, Korea). Positive samples underwent an Immunofluorescence Assay (IFA) with the *T. cruzi* IFA kit (Trinity Biotech, Ireland) to improve specificity and titer the samples (Leiby et al., 2000). Sera were considered reactive when fluorescence was observed at a 1:64 or higher dilution.

A nested Polymerase Chain Reaction (PCR) to detect *T. cruzi* Kinetoplast DNA (kDNA) was performed in the blood of the CD patients. The first reaction used the primers 121F (5´-AAATAATGTACCGGKGAGATGCATGA-3´) and 122R (5´-GGTTGGGTTGGTGTAATATA-3´) to amplify a region of 330 bp, while the second reaction used the primers T3 (5´-TCTTTGGTGTGATCGTTAC-3´) and T4 (5´-TACATTCTATTTCTTCTCTG-3´) to amplify a region of 150 bp, improving sensitivity (Lozano et al., 2023).

CD-positive patients were monitored by the Infectious Diseases Unit with a protocol similar to the proposal of Gonza´lez Martínez et al. (2024), which included physical examinations, electrocardiograms, chest X-rays, echocardiograms, barium enemas, and esophagograms. Patients with possible cardiac involvement also underwent cardiac magnetic resonance imaging and 24-hour Holter monitoring.

*T. cruzi* infection in newborns and children of CD-positive mothers was analyzed by LiaisonXLmurex chemiluminescence kit (Diasorin, Italy) and by PCR in cord blood and sera at one month of age and at 9–10 months after birth. CD-positive children were treated in the Pediatric Infectious Diseases Unit according to the Comunitat Valenciana protocol (Bayo´n Rueda et al., 2009).

Clinical and diagnostic test were performed by the co-authors.

BNZ (Abarax, Elea Laboratory, Argentina) was administered orally in two or three doses for 60 days at 5–7.5 mg/kg per day in adults and 10 mg/kg per day in children.

Continuous and categorical variables were compared using the Student's *t*-test and Pearson's $\chi2$ test respectively (IBM SPSS Statistics v12.0). *p*-values $< 0.05$ were considered significant.

Population data were obtained from the National Institute of Statistics and the Microbiological Surveillance Network of the Comunitat Valenciana (RedMIVA).

This study was approved by the HUiP La Fe ethics committee on October 10, 2017, with registration number 2016/0866. Since this study was designed as a retrospective study, no patient consent was included in the protocol. The patients of the study were registered with HUiP La Fe, but not with other govermment agency.

## 3. Results

A descriptive analysis of patients from CD-endemic areas was performed at HUiP La Fe. Out of 3713 subjects, 433 (327 adults and 106 children) had positive serology for CD by three tests at the diagnosis study, two different screening tests as recommended by WHO, followed by an IFA: 303/433 (70 %) were women and 130/433 (30 %) were men. This gives the CD prevalence in the sample of 11.7 %.

There were discrepancies in the diagnosis of 14 samples that were considered false positives. Five samples had low signal levels in IFI and chemiluminescence results, whereas nine had low signal levels in chemiluminescence and negative IFI. A subsequent serum sample was requested six months later from all 14 patients and was negative.

The mean age of individuals with positive serology was statistically different between women (37.5$\pm$10 years old) and men (40.9$\pm$12.6 years old) ($p$=0.03).

*T. cruzi* DNA detection by PCR was positive in 20 % (85/433) individuals who had CD positive serology.

### 3.1. Patients' origin

Data regarding the origin of 327 adults diagnosed positive for CD are shown in Table 1. Most patients came from Bolivia (91.1 %), followed by Ecuador (1.8 %), Argentina (1.5 %), Colombia (0.9 %), Brazil (0.3 %), El Salvador (0.3 %), and Venezuela (0.3 %).

Data on the origin of 12 patients (3.7 %) were missing because these patients did not attend the Infectious Disease Clinic for the epidemiological interview.

Among the 298 Bolivian CD patients, the most frequent department of origin was Santa Cruz (38.3 %), followed by Cochabamba (13.8 %), Chuquisaca (11.7 %), Potosí (2.7 %), Tarija (2.7 %), La Paz (1 %), and El Beni (0.3 %). For 29.5 % of the Bolivian patients, the department was unknown.

The entire pediatric population consisted of children born in Spain, except for two children born in Bolivia.

### 3.2. Pregnant women and pediatric population

Out of 313 confirmed CD-positive adults (excluding false positives), 200 (64 %) women were followed because they were of childbearing age. Of these, 90 (45 %) were pregnant and *T. cruzi* was detected by PCR in 18 of them (20 %).

Serology was performed in 87 newborns and 19 children of CD-positive mothers. Of these, 4 newborns and 3 children (6.6 %) were positive. Four cases (3.8 %) being due to vertical transmission.

Vertical transmission could not be assessed in the other three children with confirmed CD because they had been living in an endemic area

**Table 1**
*T. cruzi* infection and gender prevalence by country of origin.

| Country of origin | Total immigrants in Valencia (women%) | CD prevalence by gender (% prevalence) | | | CD at HUiP La Fe (women/ men) |
|---|---|---|---|---|---|
| | | Woman | Men | p | |
| Bolivia | 6334 (56.3) | 1323/ 3566 (20.9) | 483/ 2768 (7.6) | <0.0001 | 225/73 |
| Argentina | 5185 (50.8) | 42/2633 (0.8) | 13/ 2552 (0.25) | <0.001 | 4/1 |
| Ecuador | 6844 (44.4) | 563,038 (0.8) | 9/ 3806 (0.1) | <0.0001 | 4/2 |
| Colombia | 19,474 (55.4) | 54/ 10,788 (0.3) | 8/ 8686 (0.04) | <0.0001 | 3/0 |
| Brasil | 4765 (61.8) | 10/2944 (0.2) | 1/ 1821 (0.02) | | 1/0 |
| El Salvador | 1631 (38.3) | 6/624 (0.4) | 1/ 1007 (0.06) | 0.03 | 1/0 |
| Venezuela | 12,575 (56) | 14/7042 (0.1) | 30/ 5533 (0.02) | | 1/0 |

prior to diagnosis.

### 3.3. BNZ treatment and adverse reactions

Treatment with BNZ was implemented in 204(49 %) out of 419 confirmed-diagnosed CD patients, including 5 children and 199 adults (147 women and 52 men). More than half of the treated adult patients (54 %, 107/199) developed the adverse reactions described in Table 2.

### 3.4. Monitoring CD symptoms

Among the adult individuals with positive serology for CD, 90 did not attend the physical examination because they moved to other regions. Of the remaining individuals, 63/223 (28.3 %) presented the CD related symptoms described in Table 3. Symptomatic CD was more frequent in men than in women, 29/61(47.5 %) *vs* 34/162(21 %) [$\chi^2$(1, $N$=223) =15.4 $p$ <0.001)], although no association between sex and the type of symptoms (p 0.9) was found.

Almost half of the patients to whom an epidemiological interview was made, 49.2 % (148/301), stated that they had at least a family member with CD in their country of origin.

A total of 55/223(24.7 %) patients had some hospital admission during the study period, and 20(36,3 %) of these episodes were related to CD complications.

### 3.5. Solid organ transplantation

During this period, there were three CD patients who underwent organ transplantation; two of them had CD and received a cardiac and liver transplant, respectively. The third patient was a CD-negative liver recipient from a CD-positive donor. In the last case, follow-up was conducted after liver transplantation, and kDNA *T. cruzi* was detected by PCR 43 days post-transplantation. CD treatment with BNZ (5 mg/kg/ day) was started, and *T. cruzi* DNA was nondetectable after 38 and 91 days post-transplantation.

**Table 2**
ADRs in CD patients.

| Adverse Drug Reactions | n (%) |
|---|---|
| Dermatological symptoms | 69 (64 %) |
| DRESS syndrome | 6 (6 %) |
| Paresthesia with skin reactions | 8 (7 %) |
| Common symptoms (fever/arthralgia) | 5 (5 %) |
| Non-specific symptoms (dizziness) | 19 (18 %) |

**Table 3**
Type of CD-related symptoms in patients with *T. cruzi* infection.

| Symptoms | Total | Women ( %) | Men (%) |
|---|---|---|---|
| **Cardiac** | **39** | **21 (61.8)** | **18 (62.1)** |
| RBBB* | | 5 (14.7) | 13 (44.8) |
| Pacemaker | | 5 (14.7) | 2 (6.9) |
| Heart failure | | 2 (5.9) | 1 (3.5) |
| Valvular insufficiency | | 4 (11.8) | 1 (3.5) |
| Heart transplant | | 1 (2.9) | 0 (0.0) |
| Cardiomegaly | | 3 (8.8) | 1 (3.5) |
| Dilated cardiomyopathy | | 7 (20.6) | 3 (10.3) |
| Bradycardia | | 7 (20.6) | 1 (3.5) |
| **Digestive** | **19** | **11 (32.4)** | **8 (27.6)** |
| Dolichosigma | | 6 (17.6) | 1 (3.5) |
| Megacolon | | 3 (8.8) | 2 (6.9) |
| Megaesophagus | | 2 (5.9) | 5 (17.2) |
| Chronic constipation | | 5 (14.7) | 1 (3.5) |
| Achalasia | | 0 (0.0) | 1 (3.5) |
| **Both** | **5** | **2 (5.9)** | **3 (10.3)** |
| Bradycardia and Dolichosigma | | 1 (2.9) | |
| RBBB and Dolichosigma | | 1 (2.9) | |
| LBBB<sup>ψ</sup> and Dolichosigma | | | 1 (3.5) |
| Atrioventricular block and Megacolon | | | 1 (3.5) |
| RBBB and Megaesophagus | | | 1 (3.5) |

* **RBBB**, Right bundle branch block;.
ψ **LBBB**, Left bundle branch block.

### 3.6. Coinfections

As requested by health practitioners some CD-positive patients underwent specific serology for HIV and some parasites. The data are presented in the Supplementary material.

## 4. Discussion

Our results show that CD prevalence at the Health Department Valencia-La Fe is not negligible. An increasing number of women of childbearing age from endemic areas are diagnosed through the screening programs of the Spanish public health system, such as those implemented in the Comunitat Valenciana, Galicia, and Catalonia (Basile et al., 2019). Nevertheless, since men are not screened until they present symptoms, studies are recommended to assess the feasibility and cost-effectiveness of screening programs for men from endemic areas.

The prevalence of CD observed in immigrants in our health department was 11.7 %, while CD prevalence in non-endemic countries can vary greatly depending on the population studied. Analyzing migrant population from endemic areas, Navarro et al. (2022) estimated overall CD prevalence in Spain of 2.1 %, whereas in Europe it was estimated to be 4.2 %. Our data show a higher prevalence in a population mostly composed of Bolivians (91.2 %), but different studies yield similar values for this population (Romay-Barja et al., 2019; Velasco et al., 2020; Navarro et al., 2022).

In a systematic review, CD prevalence among immigrants can be as high as 13 % and, among pregnant women, as high as 4 %, with the vertical transmission ratio about 3 % (Velasco et al., 2020). A recent article about CD in pregnant women in Madrid showed that the highest CD prevalence was found in Bolivian women (12.4 %), while pooled prevalence was 2.9 % (Herrero-Martínez et al., 2023). Carlier et al. (2019) estimated that the vertical transmission rate in chronic CD was 4.7 %, in line with the 3.8 % we observed.

In our study, 26.6 % of all CD patients had some visceral involvement, with cardiac dysfunction being the most frequent, in agreement

with Velasco et al. (2020). Most of them were men (46 %) diagnosed at older ages than women, as observed in other studies performed in some areas of Chile (Salas, 2020). Our study also found that the colon was the gastrointestinal organ most frequently affected, as shown by Matsuda et al. (2009).

Regarding ADRs, different studies present varying results. Adverse reactions can occur in 30–87 % of patients, depending on the series, implying about 12–29 % treatment abandonment (Sperandio da Silva et al., 2014). Our study had similar results, 54 % (107/199) of the patients presented ADRs and 6 % (12/199) had to abandon the treatment.

In endemic countries, the rate of coinfection with HIV ranges from 1.3 to 7.1 % (Molina et al., 2016). Although in our study population not everyone underwent an HIV screening test, the percentage of patients with both pathologies (2 %) aligns with these studies. Regarding the study of coinfections with other parasites, serological tests for *S. stercoralis* were requested only in 84 cases with CD, but the prevalence reached 12 %. This association has been described previously and should be studied in patients from endemic areas (Puerta-Alcalde et al., 2018). Our new CD protocol follows this recommendation. The association between parasites and fewer complications deserves further studies.

Cura et al. (2013) concluded in a series of cases of organ transplantation that early *T. cruzi* PCR detection in organ transplant recipients from seropositive donors allows early treatment and consequently prevents chronic CD appearance. In our study, the only CD-positive organ recipient was diagnosed by PCR 43 days after transplantation, and *T. cruzi* DNA was nondetectable after 38 days of treatment.

Finally, one of the strengths of our study is the large number of patients included, making it one of the largest carried out in the Comunitat Valenciana. Thus, it describes the epidemiological reality of this disease in a non-endemic country. However, the absence of a protocol to detect CD in people other than women of childbearing age leaves many patients underdiagnosed and untreated, especially adult males.

## 5. Conclusion

Even though our knowledge of CD has expanded in recent years, further research is needed to fully understand this disease. Despite efforts, CD remains an unknown disease for many physicians and healthcare practitioners, and awareness of the disease is quite low. Thus, many people cannot benefit from early diagnosis. Early detection and treatment, and adequate follow-up should be mandatory for any individual with CD.

## CRediT authorship contribution statement

**Noelia Lozano:** Formal analysis, Conceptualization, Writing – review & editing, Writing – original draft, Methodology, Investigation. **Carmen Lloret-Sos:** Supervision, Validation. **María Jose´ Gime´nez-Martí:** Supervision, Validation, Writing – review & editing, Writing – original draft. **Jose´ Miguel Sahuquillo-Arce:** Supervision, Data curation, Writing – review & editing, Writing – original draft. **María Dolores Go´mez-Ruiz:** Validation, Writing – review & editing, Writing – original draft. **María Trelis:** Supervision, Writing – review & editing. **Jose´ Luis Lo´pez-Hontangas:** Writing – review & editing. **Antonio Osuna:** Writing – review & editing, Supervision. **Eva Calabuig:** Writing – review & editing, Validation, Resources, Methodology, Formal analysis.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2024.107458.

## Data availability

Data will be made available on request.

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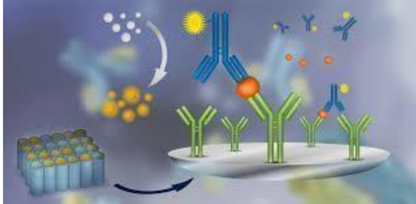
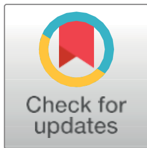


# *Circulating exovesicles in sera of chronic patients as a method for determining active parasitism in Chagas disease.*

Noelia Lozano, Alexa Prescilla- Ledezma, Eva Calabuig, María Trelis, José Miguel Sahuquillo-Arce, José Luis López-Hontangas, Luis Miguel de Pablos, Mercedes Gómez-Samblas, Antonio Osuna.



RESEARCH ARTICLE

# Circulating extracellular vesicles in sera of chronic patients as a method for determining active parasitism in Chagas disease

**Noelia Lozano[1,2☯], Alexa Prescilla-Ledezma[3,4☯], Eva Calabuig[5,6], Maria Trelis[7,8], José Miguel Sahuquillo Arce[2], José Luis López Hontangas[2], Luis Miguel de Pablos[4], Mercedes Gomez-Samblas[4‡], Antonio Osuna[4‡]****

**1** Area of Parasitology, Department of Pharmacy and Pharmaceutical Technology and Parasitology, University of Valencia, Burjassot, Valencia, Spain, **2** Department of Clinical Microbiology and Parasitology, Hospital Universitario y Politécnico La Fe, Martorell, Valencia, Spain, **3** Department of Human Microbiology, Faculty of Medicine, University of Panama, Panama, **4** Department of Parasitology, Biochemical and Molecular Parasitology Group CTS-183, and Institute of Biotechnology, University of Granada., Granada, Spain, **5** Infectious Diseases Unit, Internal Medicine Department, Hospital Universitario y Politécnico La Fe, Martorell, Valencia, Spain, **6** University of Valencia-Health Research Institute La Fe-IIS, Valencia, Spain, **7** Area of Parasitology, Department of Pharmacy and Pharmaceutical Technology and Parasitology, University of Valencia, Burjassot, Valencia, Spain, **8** Joint Research Unit on Endocrinology, Nutrition and Clinical Dietetics, University of Valencia-Health Research Institute La Fe-IIS, Valencia, Spain

☯ These authors contributed equally to this work.
‡ These authors also contributed equally to this work.
* aosuna@ugr.es

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## Abstract

### Background

Chagas disease, once restricted mainly to the Americas, Chagas disease has become a global health problem due to migration from endemic to non-endemic areas. In non-endemic regions, transmission is limited to vertical transmission from infected mothers to newborns or through blood and organ donations. A major challenge in the management of the disease lies in the diagnosis of chronic cases, as blood-borne parasites are often absent and antibodies persist for life, complicating the evaluation of treatment.

### Methodology and main findings

This study investigates whether detection of circulating extracellular vesicles (EVs) or their immunocomplexes with host IgGs in the serum of chronic patients with Chagas disease could serve as diagnostic tools and biomarkers of the active presence of the parasite. This method may prove valuable in cases where parasitaemia and other diagnostic tests are inconclusive, especially for assessing treatment efficacy and confirming mother-to-child transmission. Together with exovesicle purification by ultracentrifugation, which is the 'gold standard', an affordable and simplified method for the isolation of EVs or immunocomplexes was tested for use in less well-equipped diagnostic laboratories.

EV detection was performed by enzyme-linked immunosorbent assay (ELISA) targeting Trypanosoma cruzi antigens. Positive results were demonstrated in Bolivian patients in Spain, covering asymptomatic and symptomatic cases (cardiac, gastrointestinal or both).

"Exovesiculas circulantes como marcadoras de diagnostico PREcoz de la Enfermedad de CHAGas" to LMP and AO; Spanish Agency of Science Exovesicles of Trypanosoma cruzi and the immunocomplexes that they form. Implications in the pathology of Chagas disease PGC2018-099424-B-I00 to AO and I+D+i Proyect "Programa Operativo FEDER de Andaluc´ıa JJAA" 2014-2020 A-BIO-350-UGR18 to LMP. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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The study also examined infected mothers and their newborns. These findings were further confirmed in Panamanian patients with inconclusive diagnostic results.

Moreover, host IgG isotypes that formed immunocomplexes with parasite exovsicles were identified, with IgG2 and IgG4 being predominant.

## Conclusions

Our results confirm the usefulness of circulating EVs and their immunocomplexes as markers of metabolically active T. cruzi in chronic infections without detectable parasitaemia, as well as their efficacy in confirming vertical transmission and in cases of inconclusive diagnostic tests.

## Author summary

Chagas disease (CD), or American trypanosomiasis, is considered by the WHO as one of the most important neglected diseases of the American continent, considered as strictly American, there are currently cases of the disease spread all over the world as a consequence of human movements even in areas where the biological cycle of the protozoan parasite responsible for the disease does not occur.

Laboratory diagnostic methods vary between direct tests that denote active parasitemia in blood in the acute phase of the disease and indirect molecular methods applied when the circulating parasitemia disappears in the chronic phase of the disease.

Despite the existence of numerous diagnostic, molecular or immunological tests, the latter being generally easier to perform in diagnostic laboratories, the presence of the parasite always remains in doubt in various situations. Particularly in those patients after treatment since the presence of antibodies to the parasite remain at high titres for years, or in the case of newborn diagnosis in which the mother's IgG makes specific immunological tests impossible for months.

Therefore, given the need to find biomarkers in chronically ill patients or in mothers and newborns that indicate the active presence of parasites, we have used the purification of parasite extracellular vesicles (EVs) in circulating blood or the immunocomplexes they form with host IgGs, by means of two techniques, the gold standard technique by ultracentrifugation and an adapted technique, easier to apply in diagnostic laboratories, to purify these EVs in order to study the recognition of these EVs by antibodies against the parasite or against specific antigens of *Trypanosoma cruzi* specific antigens.

We believe that this technique can be used when it is necessary to evaluate the efficacy of treatments or in neonatal diagnosis.

## Introduction

Chagas disease (CD), or American Trypanosomiasis, is considered by the World Health Organization (WHO) to be one of the most important neglected diseases in the Americas [1,2], where about 8 million people are affected, with about 15,000 deaths per year due to this disease [3,4].

Until a few decades ago, the disease was considered a strictly American disease, endemic in 21 countries. From southern Texas and New Mexico to Argentina and Chile, today, due to

human migratory flows, it is considered to have a worldwide distribution [5]. Cases have been diagnosed in geographical areas where epidemiological conditions do not allow the natural maintenance of the disease, such as North America (central and northern USA and Canada); Europe (countries where the migratory flow from Latin America has been important, especially in Spain, Italy, Sweden), and Australia. Considering that in Spain alone, the number of people affected within the Ibero-American population is estimated at around 55,000 cases [5,6].

CD is caused by the protozoan *Trypanosoma cruzi*, a flagellated protozoan which, in addition to humans, affects domestic and wild mammals, some of which act as reservoirs, and CD should therefore be considered a zoonosis. Hematophagous insects belonging to the family Triatominae (Reduviid) are responsible for the transmission of CD, acting as invertebrate hosts or vectors in the natural transmission of the disease. The flagellate, once ingested with the blood of the infected mammal, multiplies in the midgut of the vector in the form of epimastigotes, which eventually reach the end of the intestine and the rectal ampulla giving rise to metacyclic trypomastigotes or mammal-infective forms.

The development of the disease has a series of distinct stages, an incubation phase, lasting one week to 20 days depending on the vectorial or transfussional route of infection. The incubation phase is usually asymptomatic and lacks specific pathognomonic symptoms. The acute phase occurs in the first weeks after infection with a high blood parasitaemia characterising this acute phase. The acute phase disappears and with it the circulating parasitaemia, approximately three to eight weeks after infection, leading to the chronic phase (Chronic Chagas disease, CCD) which will last for decades with mild to no symptoms and especially with a very low level of parasitaemia. It is estimated that only 30% of cases develop pathognomonic symptoms of the disease [7–9]. The symptoms include cardiac and/or gastrointestinal disorders [10]. These chronic patients (CCD) constitute the greatest epidemiological risk of disease transmission in countries where infection by insect vectors does not occur.

Laboratory diagnostic methods vary from direct tests that denote the active presence of the parasite, such as microscopic observation of the parasite in blood, the microhaematocrit buffy coat [11,12] to xenodiagnosis using triatomines fed on patients and applicable to both the acute and chronic phases of the disease, sometimes including the use of PCR to detect the active presence of *T. cruzi* in the gut of the insects used for diagnosis [13], methods rarely or not at all used in in non-endemic countries, or more commonly the use of indirect techniques as different immunological techniques, from rapid immunocomatography tests, indirect immunofluorescence (IIF), enzyme-linked immunosorbent assays (ELISA) with different antigens, Western Blotting (WB), or more recent procedures such as chemiluminescent techniques in which different antigens (native or recombinant) or chimeric antigens are used [14,15].

The variability in diagnostic accuracy has been related not only to the type of technique or antigen used, but also to geographical differences in infected patients, differences in infecting parasite strains or the origin of the diagnostic antigen, or genetic differences between human populations, which may contribute to discrepancies in the sensitivity and specificity of different serological tests [16]. These controversies have led to a series of recommendations by the Pan American Health Organisation (PAHO) and national guidelines [17–19] recommending the confirmatory use of two serological tests in parallel, with a sensitivity of at least 98% [20] or a correct diagnosis of the disease. On the other hand, in treatment efficacy studies or in the case of neonatal diagnosis, it is necessary to demonstrate the active presence of the parasite either by tests that allow visualisation, isolation and growth of the parasites or by other unequivocal tests that show the active presence of parasites in the patient's biological fluids such as blood, serum or plasma.

The presence of immunocomplexes in patients with CD has been described by some authors both in chagasic patients and in experimentally infected animals [21], attributing some of the pathological manifestations of the chronic phase of the disease to such immunocomplexes [22,23]. Some publications have considered these immunocomplexes as useful tools for diagnosis. For instance, Ohyama et al. (2016) [24] and Petray et al. (1992) [25] studied the parasite antigens present in the serum immunocomplexes of patients affected by CCD using proteomic analysis. In their results, the presence of proteins such as Transialidases or GP63, proteins typical of trypanosomatids (*T*. *cruzi*, *Leishmania* ssp. or *Trypanosoma brucei*), was found [26–28]. D´ıaz-Lozano et al. (2017) [29] described how these immunocomplexes can be formed by extracellular vesicles (EVs) from the parasite and immunoglobulins from the host, these immunoglobulin-linked EVs were present in the serum of chronic chagasic patients regardless of the pathology of the patients and how these immunocomplexes could be prognostic markers of disease pathology, acting as carriers for a series of parasite-specific proteins, and without orthologues in other Trypanosomatids, such as proteins belonging to the mucin-associated surface proteins (MASP) family. MASP is a multigene family of approximately 1,300 proteins [30–32] which have a high variability, except in two regions of identical nucleotide sequences in all the proteins of the multigene family, the region corresponding to the C-terminal 5' sequence (C-term) that is coding for a signal peptide (SP), and the N-terminal 3' region. The rest of the sequence is hypervariable which makes the MASP proteins different from each other [33].

EVs are small membrane-coated vesicles released into the extracellular environment by all types of cells, both eukaryotic and prokaryotic, and are classified according to their size, biogenesis and composition, including exosomes (~30–100 nm), ectosomes (~100–500 nm) and apoptotic vesicles ($>$ 500 nm) [34]. EVs act as carriers including a wide variety of lipids, proteins, different populations of RNAs, ssDNA, and/or metabolites. EVs participate in cell-cell communication processes in an endocrine, paracrine or juxtacrine manner [35]. They can participate in numerous cellular functions from immunomodulation, antigenic presentation [36], modify cellular niches or be carriers of genetic markers and gene transfer between cells [37] and can be useful in molecular diagnostic systems [38].

The production of EVs by *T*. *cruzi* was first described by Da Silveira et al. (1979) [39] and the role of these EVs in promoting parasitism has been demonstrated, [40–42] both at organ and cellular level, due to their ability to induce changes in the cells which they interact [43] modulating cell physiology such as the cytoskeleton of cells, modifying cytosolic calcium levels, altering the permeability of cells, modifying the cell cycle or the transcriptome [44,45]. Proteomic studies of EVs released by trypomastigote forms show a series of parasite-specific proteins such as MASP proteins, or transialidases, which together with cruzipain constitute specific antigens capable of being recognised by the immune system of affected patients [29,46–49].

As EVs constitute specific carriers of both proteins and nucleic acids, Lozano et al. (2023) [48] recently determined how EVs from the plasma of chronic CD patients could be used in the molecular diagnosis of Chagas disease, demonstrating how these EVs carry nucleic acids from the parasites and can be amplified using either strictly mitochondrial KDNA-specific probes or nuclear DNA probes capable of amplifying parasite satellite DNA. This fact demonstrates that in these chronic patients, with little or no circulating parasitemia by traditional techniques, there must be metabolically active forms of the parasite, such as trypomastigote or amastigote forms, capable of releasing EVs, we have evaluated the possibility of using EVs derived from the parasite or forming circulating immunocomplexes in the serum of patients with CCD from two different geographic regions of America, as new diagnostic biomarkers that denote the active presence of *Trypanosoma cruzi*.

The aim was: i) to determine whether the detection of circulating EVs present in the sera of chronic patients with Chagas disease, typed and clinically classified according to symptomatology, contain circulating immunocomplexes formed by parasite EVs and IgGs of the host, denoting the biochemically active presence of the parasites; ii) we also attempted to characterize the different isotypes of IgGs that form these immunocomplexes, which may help in the future to purify these immunocomplexes by immunochromatographic techniques; iii) we tried to develop an easy and affordable technique for the isolation of immunocomplexes, which avoids having to use exovesicle purification by ultracentrifugation (the gold standard) and which can be applicable in diagnostic laboratories where specialized instrumentation is lacking; iv) we tried to develop a technique to be used in those cases where it is required to demonstrate the presence or absence of metabolically active parasites, as in the case of analysis of the efficacy of treatments or newborns where traditional techniques for the study of parasitemia encounter difficulties due to the low number of parasites in blood or other biological fluids.

The detection of EVs was performed by immunological techniques using either an immunoserum against *Typanosoma cruzi* or antibodies against highly parasite-specific proteins such as the signal peptide of the MASP proteins, a region of constant sequence in this large family of proteins.

# Materials and methods

## Ethics statement

For the study of Bolivian patients residing in Spain, we have the permissions of the following Ethics Committees: Ethics Committees of HUyP-La Fe with the numbers HUyP-La Fe, Valencia, Spain (2016/0866), and of the University of Granada, Human University of the Granada Ethical Committee, Spain, with number n˚: 672/CEIH/2018.

All participating patients received an 'informed consent' document to be signed, which is included in the supplementary material. The document includes a section for the "clinical study in children", where the notification to the Public Prosecutor's Office is included, as required by Spanish legislation for the participation of minors, and which must be signed by the parents or legal guardians of the minors.

For the study of the patients from Panama, permission was obtained from the Ethics Committee of the University of Panama, Faculty of Medicine with number 2015-310V1 and from the Human Ethics Committee of the University of Granada with number 672/CEIH/2018. All Panamanian patient participants received an informed consent document which they were required to sign in accordance with Panamanian regulations.

## Study populations

In this study, two populations of patients with CCD from Latin America were examined.

The first population comprised 92 patients of Bolivian origin who currently reside in Spain. These individuals live in the Spanish city of Valencia, where they were diagnosed and underwent medical follow-up at the Hospital Universitario y Politécnico La Fe (HUyP-La Fe) Valencia, Spain. The Bolivian samples included 92 patients who underwent screening tests, applying three serological assays: i) LiaisonXL murex (Diasorin), ii) rapid test (SD Bioline Chagas Ab Rapid, Abbott 49FK10), and iii) IFA kit (Trinity Biotech). All these tests, as mentioned earlier, are routinely applied for the diagnosis of individuals suspected of being affected by CCD at the HUyP-La Fe. Of the 92 Bolivian patients, 63 were adults with CCD, comprising 49 (77.8%) females and 14 (22.2%) males. Additionally, there

were 16 newborns from mothers with CD and 13 CD-negative individuals, selected as controls.

The 63 patients with positive immunological tests or PCR (Lozano et al 2023) were summoned to the Infectious Diseases Unit, Internal Medicine Department, for control and monitoring of CD. A physical examination, an electrocardiogram, a chest x-ray, an echocardiogram, a barium enema and esophagogram were performed and it was registered whether they presented any cardiac or digestive symptoms at the hospital electronic medical chart. Patients with possible cardiac involvement also underwent cardiac magnetic resonance imaging and 24-hour Holter monitoring. Of these of the adult patients, 24 had indeterminate symptoms, including 16 pregnant women. There were 20 patients with cardiac involvement, 14 with gastrointestinal pathology, and 5 with both cardiac and gastrointestinal symptoms. In the case of the 16 newborns, samples were analyzed by PCR at birth, repeated at one month and at nine months of age.

The second group, and as a proof of concept of the results obtained with patients diagnosed in Spain, consisted of 106 individualized sera of CCD patients, coming from Panama a country considered endemic for CD since 1930. The samples had been used in a previous work by our research group [50] and in which the DTU of the parasites that produced the infection had been typed. Sampling was conducted in both rural areas, where patients have continuous contact with vector insects, and urban areas within Panama City. Informed consent procedures, surveys, and blood sample collection were carried out for all patients who voluntarily chose to participate in the study. Of the studied Panamanian population, a total of 106 potential CD patients were analyzed, with 78 out of the 106 (73.58%) residing in the rural community of Charare´, located in the mountainous region of the Las Margaritas, Chepo district, Panama province (coordinates 9.243640, -79.059162). Screening was conducted using three serological tests: i) rapid test (SD Bioline Chagas Ab Rapid, Abbott 49FK10); ii) WB using the methodology previously described by Saldaña et al. (1995) [51]; iii) ELISA Chagatest (Wiener lab 1293257) [50]. Only individuals who tested positive in two out of these three tests were considered positive. Out of the initial 106 individuals, 53 were identified as positive (33 from rural areas and 20 from urban areas) and were used to validate the presented diagnostic methods. A selection of 25 sera randomly selected, from the individuals previously diagnosed as positive in Panama [50] by screening with at least two of the serological tests described above and clinically evaluated in the hospitals of Chepo and Santo Tomas, after an electrocardiographic examination and thoracic radiography and clinically classified as Chagas positive. In all cases, negative parasitization by *Trypanosoma rangeli* and *Leishmania ssp*. was evaluated, which in the case of Panamanian leishmaniasis is of cutaneous pathology. In order to calculate the cut-off value of the absorbances obtained from the circulating Evs in the sera of the patients. Fifty sera were used as shown in S3 Table, from individuals negative by immunological and PCR tests for CD, from the same geographical region. Of these sera, five showed syphilis pathology and two showed leprosy pathology. None of these cases showed infection by Leishmania ssp. or *T*. *rangeli*.

For the validation of immunocomplexes detection after dissociation of the immunoglobulins present in the sera of CCD patients, a total of 117 sera from both patient populations were used. This included 92 sera from the population diagnosed at HUyP-La Fe, and a selection of 25 sera from the 53 individuals previously diagnosed as positive in Panama through screening with the three serological tests described above. This selection included two sera negative for ELISA Chagatest, two negative for WB, two negative for the rapid test, one negative for both ELISA Chagatest and WB, 16 positives for all three screening tests, plus a negative reference serum and an existing positive reference serum in our laboratory's serum bank.

## Immunological tests used for Chagas disease patient diagnosis

As previously mentioned, the immunodiagnostic tests employed for diagnosing patient populations varied based on their availability at the hospitals where the patients were recruited and analyzed.

For patients of Bolivian origin diagnosed at HUyP-La Fe, analysis was conducted using the LiaisonXL murex kit (Diasorin). This kit employs a chemiluminescence immunoassay with recombinant antigenic proteins (multi-antigen). The methodology followed was in accordance with the manufacturer's recommendations. As a second diagnostic test, an immunofluorescence assay (IFA) kit (Trinity Biotech) was used, following the manufacturer's guidelines.

For the diagnosis and confirmation of the Panamanian patients, the initial test used was the rapid test (SD Bioline Chagas Ab Rapid, Abbott 49FK10). To conduct the assay, 100 μL of serum were deposited into the sample well along with 50 μL of assay buffer. Test interpretation was performed visually after a 15-minute incubation period at room temperature.

Another diagnostic test used for CD diagnosis in this population was a commercial ELISA test for antibodies against *T. cruzi*, specifically the ELISA Chagatest (Wiener lab 1293257). The assay was carried out and validated following the manufacturer's instructions; accordingly, serum samples were diluted to a concentration of 1:20.

All serum samples from Central American patients underwent an antigen recognition test for the parasite by patient immunoglobulins using WB, following the methodology described by Saldaña et al. (1995) [51] and subsequently published by Ledezma et al. (2020) [50]. For diagnostic use, the transferred strips were incubated with patient serum (1:100) for 2 hours. Subsequent treatment after the washes was done with peroxidase-conjugated rabbit secondary antibodies (Dako, anti-Human IgA, IgG, IgM, Kappa, Lambda/HRP, ref: P0212) diluted 1:700 in PBS, for 1 hour, as previously described [33].

## Isolation of immunocomplexes by ultracentrifugation "Gold Standard" and dissociation of purified immunocomplexes (Ig-EVs)

The purification of circulating immunocomplexes (Ig-EVs) in the serum of patients was carried out following methods previously described by D´ıaz-Lozano et al. (2017) [29], and Lozano et al. (2023) [52] through a mixed procedure of filtration through 0.45 μm filters followed by differential ultracentrifugation at 110.000 xg for 2h in in microcentrifuge tubes (Hitachi No 1508) at 110,000 × g for 2 hours at 4 ˚C in a CP100NX centrifuge (Hitachi Koki, Tokyo, Japan) with a fixed-angle rotor P70A.

After this centrifugation stage, the pellets containing the immunocomplexes were washed three times by ultracentrifugation in sterile filtered PBS and evaluated through nanoparticle tracking analysis (NTA) and transmission electron microscopy, as described in Lozano et al. (2023) [52] and Cornet-Gomez et al 2023 [53].

In order to separate the EVs present in the sera from the immunoglobulins forming the immunocomplexes, the pellet containing the immunocomplexes was resuspended in 90 μl of PBS containing a cocktail of protease inhibitors without EDTA (Roche, ref: 11836170001). Subsequently, to the suspension containing the immunocomplexes, 650 μl of 0.1 M glycine-HCl at pH 4 were added and incubated for 15 minutes at room temperature. This suspension in glycine-HCl pH 4 buffer was ultracentrifuged again at 100,000 x g for 1 hour to separate circulating EVs in the pellet and the immunoglobulin solution that forms the immunocomplexes in the supernatant. The supernatant was aliquoted and pH-neutralized with Tris-HCl Buffer, pH 10, Antigen Retriever (Sigma T6455) containing 0.1% Glycerol, frozen, and kept at -20 ˚C until use for IgGs purification by affinity chromatography using Protein G and subsequent determination of the isotype, as described later. The pellet obtained from ultracentrifugation

was resuspended in PBS, centrifuged again at 100,000 x g, the supernatant removed, and resuspended in 80 μl of 0.1 M bicarbonate buffer (pH 9.6) containing protease inhibitors without EDTA (Roche, ref: 11836170001).

## Isolation and concentration of EVs through filtration with centrifugal concentrators

As an alternative method, for the concentration of EVs and immunocomplexes present in serum in order to avoid using the ultracentrigugation techniques described above, which may be unaffordable for hospital and health center laboratories, the method using protein concentrators previously described by Orrego et al. (2021) [54] and Ram´ırez et al. (2018) [55] was followed, with some modifications.

Briefly, patient serum (1 ml) was diluted with 5 ml of ultrafiltered PBS. Subsequently, centrifugation at 1,500 x g for 10 minutes was conducted, and the resulting supernatant was filtered through a pore size of 0.45 μm. Following this initial filtration and centrifugation, the filtered supernatant underwent a further centrifugation step at 3,500 × g for 20 minutes. The final supernatant, diluted with PBS was applied to Vivaspin protein concentrator (Sartorius Lab Instrument, Goettingen, Germany) with a separation cut-off size of 100K (100,000 MWCO), which were centrifuged at 6,000 × g for 1 hour at 4 ˚C. The retained volume in each concentrator was collected, aliquoted, and stored at -80 ˚C until use.

## Determination of the size of EVs and purified immunocomplexes

The hydrodynamic size distribution of the purified immunocomplexes obtained by either method described above was measured by NTA (Nanoparticle Tracking Analysis), using an instrument equipped with a sample chamber, a 405-nm laser, and a high-sensitivity complementary metal-oxide-semiconductor (CMOS) camera. The samples were diluted in 0.22 μm filtered PBS up to 1 ml and then loaded into the chamber. Three 60 s videos, in Brownian mode, were recorded and analyzed for each sample with NTA 2.3 image-analysis software (NanoSight Ltd., Amesbury, UK). The mean size distribution was calculated as a mean of three independent size distributions. This methodology follows the procedures previously described by Retana-Moreira et al. (2021) [48] and by Lozano et al. (2023) [52] and by Cornet-Gomez et al. (2023) [53].

## Use of animals for the Production of antisera and authorization by the animal welfare and ethics committee

The use of animals for obtaining antisera was carried out in accordance with the guidelines set forth in the Spanish Government Regulation (Royal Decree RD1201/05) and the European Union Directive (European Directive 2010/63/EU). It was approved by the Ethics Committee of the University of Granada and by the Regional Government authorities of Andalusia (Junta de Andaluc´ıa) with the number ES180210000038 in 2017.

## Preparation of polyclonal antibodies against *T. cruzi*

Three four-week-old male Wistar rats were intraperitoneally immunized with 20 μg of a total extract from *T. cruzi* Pan4 trypomastigotes per dose, combined with Freund's adjuvant, to produce polyclonal anti-*T. cruzi* antibodies. The parasite extract was derived from $10^9$ trypomastigotes obtained from cell cultures, which were previously washed and concentrated by centrifugation, following the procedure described by Cornet et al. (2023) [53].

Antibody titers in serum samples were determined on a weekly basis after the first two immunizations using an indirect ELISA. At the end of the immunization period (8 weeks), the animals were euthanized in an isoflurane atmosphere. Whole blood samples were obtained by cardiac puncture.

To design the synthetic peptide corresponding to the consensus sequence of the signal peptide (SP) of MASP proteins, the methodology described by Díaz-Lozano et al. (2017) [29] was followed. A consensus sequence (MAMMMTGRVLLVCALCVLWSVAADG) (S3 Fig) was used, which was synthesized by LifeTein (USA, LLC) with four branches joined by lysine residues.

The production of polyclonal antibodies against the synthetic peptide corresponding to the signal peptide sequence of MASP proteins was carried out in three four-week-old male Wistar rats with 100 μg of the MASP SP peptide per dose, respectively. Before the first immunization step, a blood extraction was performed in all cases to obtain preimmune control serum. Antibody titers of anti-MASP SP and anti-*T*. *cruzi* extract sera were verified by an indirect ELISA in multiple well microtiter plates (Nunc, Thermo Fisher) coated with 5 μg of the antigen/well in 0.1 M bicarbonate coating buffer (pH 9.6). Sera with titers greater than 1:6,400 were selected and stored at -80 ˚C, diluted 1:1 with glycerol (Molecular Biology grade, Sigma) until use, and were referred to as anti-MASP SP antisera or anti-*T*. *cruzi* total antisera.

## Electrophoretic confirmation of *Trypanosoma cruzi* antigens present in circulating EVs in the plasma of patients

In order to recognize the *T*. *cruzi* antigens present in the EVs isolated from the plasma of patients affected by Chagas disease, the western blot methodology described in Retana Moreira et al [26] was followed, for which the purified EV proteins were precipitated in acetone at -20 ˚C overnight. They were centrifuged at 13,000 x g for 10 min at 4 ˚C and washed twice with cold acetone. After the acetone residue was removed, the precipitated proteins were quantified using the Micro BCA Protein Assay Kit (Thermo Scientific, ref: 23235). 30 μg of these proteins were loaded into the wells of 12% SDS-PAGE gels after electrophoresis and subsequent transfer to PVDF membranes (BioRad, Hercules, CA, USA). The membranes were immersed in blocking buffer (PBST plus 4% skimmed milk). Incubated at 4˚C for 12h with a 1:1000 dilution of polyclonal anti-*T*. *cruzi* antibody, the membranes were washed and incubated with a secondary antibody anti rat (1:10000) peroxidase-conjugated goat antibody (Sigma-Aldrich, ref: A9037) for 1h at room temperature. Detected bands were visualized using Clarity ECL Western Substrate (BioRad, Hercules, CA, USA) on a ChemiDoc Imaging system (BioRad, Hercules, CA, USA).

## Antigenic recognition by ELISA by anti-MASP SP or anti-*T*. *cruzi* immunosera from EVs isolated from patient sera

For ELISA assays, Nunc 96 multi-well plates (Thermo Fisher Scientific) with a volume of 100 μl per well were coated with a concentration of 5 μg/μl of proteins from a lysate of EVs, in RIPA buffer, isolated from each serum sample in 100mM carbonate/bicarbonate buffer (pH 9.6). The protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Scientific, ref: 23235), following the instructions. Plates were incubated under shaking for 8 hours at 4 ˚C. After adsorption, the plates were twice washed with PBST to remove unbound antigens.

Subsequently, 250 μl of a freshly prepared blocking solution were added and incubated at 4 ˚C under shaking overnight. The plates were washed again with PBST. Then, 100 μl of rat *T*. *cruzi* primary serum (1:2000) or anti-MASP SP (sera with titters higher than 1:6400) diluted

1:500 in PBS were added to each well, and the plates were incubated for 2 hours under shaking at room temperature. After antibody interaction, the plates were washed at least three times in PBST, and 100 µl of peroxidase conjugated polyclonal goat anti-rat IgG (Sigma-Aldrich, ref: A9037) at a dilution of 1:1000 in PBS were added, followed by incubation at room temperature under shaking for 1 hour.

Following incubation with the secondary antibodies, the plates were washed four times, and O-phenyl-diaminobenzidine plus 30% $H_2O_2$ (1 µl/ml) (Sigma-Aldrich) was added to 0.05 M phosphate-citrate buffer, pH 5.0, as a peroxidase substrate. The plates were further incubated for 15 minutes at 27 ˚C. The reaction was halted with a solution of 0.1 M 2 N $H_2SO_4$, and absorbance was measured at 492 nm using an ELISA Multiskan Spectrum reader (Thermo Fisher Scientific).

To determine the cut-off value of the EVs, these were individually purified from the plasma of Chagas disease-negative 13 control sera of the same origin and geographic region that were challenged by ELISA with anti-MASP SP and anti-*T*. *cruzi* immunosera, as described in the previous paragraph. And where, three negative sera from Spain, two positive for *Leishmania infantun* and one positive for leprosy, diseases in which cross-reactions with CD have been described, were included.

The cut-off value was calculated as the average OD at 492 nm plus three times the standard deviation value of the samples (mean of the OD + 3 x SD).

## Isotyping of immunoglobulin G (IgG) forming immunocomplexes with EVs isolated from CD-Positive plasma

IgGs were separated from the immunocomplexes by treatment with 0.1 M glycine-HCl at pH 4 and subsequent ultracentrifugation as described above. The IgGs were purified by chromatography using Protein G HP SpinTrap/Ab Spin Trap columns (GE Healthcare Life Sciences, 28-9031-34), following the manufacturer's instructions. Briefly, after removing the storage buffer from the columns, the column was equilibrated with 600 µL of binding buffer composed of 20 mM sodium phosphate (Sigma-Aldrich, 255793) in PBS. Washed twice with 600 µL of binding buffer to remove those sample components not bound to protein G from the column, the columns were eluted by centrifugation for 2 min at 100xg in 40 0ul of elution buffer composed of (0.1 M Glycine-HCl, pH 3.0). The columns were eluted in 2 ml vials containing 15 µL of basic pH buffer (Tris-HCl buffer, pH 10, Antigen Retriever). Elution as in the previous steps was performed by centrifugation at 100 × g for 2 minutes, repeated twice, resulting in a final eluate volume of 800 µL for each of the immunocomplex samples from each of the sera.

Once the purified IgGs were obtained, they were quantified using the Micro BCA Protein Assay Kit (Thermo Scientific, ref: 23235).

Isotyping of different IgG isotypes, as well as immunoglobulin subclasses, was performed via ELISA following the previously described methodology. Primary antibodies included IgG immunoglobulins (1:1,000) from rat (anti-human IgG2a ThermoFisher), mouse (anti-human IgG1 and anti-human IgG2 of Sigma, anti-human IgG2b of Biomedicals, and anti-human IgG3 and anti-human IgG4-HRP of Abcam) were incubated for 1 hour at room temperature under shaking. As a secondary antibody labeled with HRP peroxidase (except for IgG4, which is already labeled with HRP), a 1:1,000 dilution of anti-rat (Sigma-Aldrich, ref: A9037) or anti-mouse (Dako, ref: P0447) was added and incubated for 1 hour at room temperature with gentle stirring.

Washes were performed as described above with PBST, and 100 µl of peroxidase as substrate and incubated for 20 min at room temperature under shaking in the dark. Finally, 50 µl

of 3M HCl stop solution in distilled water were added, and the reading was carried out at 492 nm in a MultiskanSpectrum spectrophotometer (Thermo Fisher Scientific).

### Statistical analysis

The Shapiro test was used for testing normality of the distribution of the data. Normal distributed data are expressed as mean (± standard deviation) and were compared with the ANOVA and Tukey test. Nonparametric data are expressed as median (interquartile range) and were compared with the Mann-Whitney U test and the Kruskal-Wallis test. Nonparametric related data was studied with the Wilcoxon signed-rank test and the Bonferroni-corrected significance level method was applied.

Statistical analyses were performed with Rstudio and a $p < 0.05$ was considered significant.

## Results

### Sample selection. CCD patients of Bolivian origin living in Spain

Of the patients of Bolivian origin diagnosed at HUyP-La Fe, all were positive in the three immunological techniques mentioned (LiaisonXL murex (Diasorin), rapid test (SD Bioline Chagas Ab Rapid, Abbott 49FK10), IFA kit (Trinity Biotech); hence they were used in the subsequent studies (S1 Table). Of these, 63 were randomly selected from various situations and pathologies, including indeterminate symptoms, cardiac pathology, gastrointestinal disturbances, and both cardiac and gastrointestinal disturbances. Additionally, 16 pregnant women with CD and their newborns were included (summary in S2 Table).

### Ultracentrifugation vs Filtration for the detection of *T. cruzi* Antigens in EVs from Sera of CCD patients

The protein concentrator method used was designed, as already indicated, to facilitate the purification and concentration of EVs from serum or plasma in order to facilitate instrumentally and technologically the collection of EVs from serum. We compared the use of protein concentrators to purify EVs from plasma with the gold standard method for the purification of EVs (ultracentrifugation).

The results obtained from the characterization of the NTA purified EVs with both methods are shown in S1A and S1B Fig and in Table 1.

With the method in which protein concentrators were used, as shown in the Table 1, larger peaks were obtained, possibly aggregates of the EVs with each other (S1A and S1B Fig). The total protein concentration was statistically higher when the ultracentrifugation method was used for purification (27.9 ± 10.8 μg/μl) compared to the alternative filtration method (9.5 ± 8 μg/μl) (p-value <0.00001) (S2 Fig).

In order to test the antigenic recognition in EVs purified by protein concentrators and the gold standard of EV purification by the two immunisera (anti MAM Sp and anti Tc (total)), 24 individual serum samples were randomly selected from a total of 63 clinically characterized positive samples for CD from patients residing in Spain.

**Table 1. Results of the determination of the size and amount of EV protein obtained by the two purification procedures.** (*) (p-value <0.00001).

| Method | Size mean (nm) | Mode | D90 | Protein concentration (ug/ul) (*) |
|---|---|---|---|---|
| Ultracentrifugation | 209.8 | 166.5 | 290.2 | 27.9 ± 10.8 |
| Filtration | 240.4 | 208.4 | 378.1 | 9.5 ± 8 |

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Among these 24 samples individually selected samples, there were 7 pregnant women. Of the 24 samples, (S1 Table), 14 patients had indeterminate symptoms, while 5 had gastrointestinal symptoms and 5 had cardiac pathology. Each sample was treated by both ultracentrifugation-ultrafiltration and concentrator purification to separate circulating vesicles, as described in Materials and Methods.

Purification of EVs by ultracentrifugation and detection with anti-MASP SP detected those 21 patients had relative absorbance values above the cut-off threshold, while for the remaining three, the absorbance was close to 0. This same pattern was replicated when the EVs were purified by the filtration method (Fig 1A).

Employing the anti-*T*. *cruzi* for EV detection, 23 patients showed absorbance values above the cut-off threshold when EVs were purified through both ultracentrifugation and filtration (Fig 1B).

For the same EV isolation method, no significant differences were found between the two immunosera used. The Wilcoxon rank sum test yielded W = 208.5 and a p-value $>0.05$ for the ultracentrifugation method, and W = 374 with a p-value $>0.05$ for the filtration method.

For both markers, absorbance values were significantly higher when applying the ultracentrifugation technique to purify EVs from serum samples of CCD patients compared to purification via the filtration technique. Specifically, anti-*T*. *cruzi* (W = 94, p-value $<0.05$) and anti-MASP SP (W = 99, p-value $<0.05$) showed ELISA elevated absorbance levels. These findings were derived using the Wilcoxon rank sum test with continuity correction, as the variables did not adhere to a normal distribution. This suggests a consistent trend favoring the ultracentrifugation method over filtration in both markers (Fig 1A and 1B).

## Determination of antigenic recognition in EVs from Sera of CCD patients resident in Spain by Immunosera, anti-*T*. *cruz*i and anti-MASP SP

The patients were categorized into groups based on their symptoms: indeterminate symptoms (24 total patients), cardiac pathology (20 total patients), gastrointestinal symptoms (14 total patients), and those presenting with both cardiac and gastrointestinal pathologies (5 total patients).

Of the 24 samples from indeterminate patients, EVs reacted in 23 (95.8%) samples against anti-*T*. *cruzi*. The absorbance levels were under the cut-off value in a 42-year-old man (number 7), who had a previous positive PCR in 2010 and was treated, reactivity of EVs obtained to both immunosera gave absorbance values below the cut-off value (Fig 2A and Table 2).

When anti-MASP SP immunoserum was used against EVs, 20 of the 24 indeterminate patients samples (83%) had absorbances higher than the cut-off value. The absorbance levels were lower than the cut-off value in four patients, number 7 and 13 had positive PCRs and was treated, number 17 and 53 had a negative PCR and number 53 was a pregnant woman (Fig 2A and Table 2).

Of the 39 symptomatic CD patients, 35 (89.7%) samples were positive with anti-*T*. *cruzi*, and 32 (82,1%) samples with anti-MASP SP (Table 2).

Two cardiac patients (patients numbers 16 and 18), 1 gastrointestinal (patient number 45) and 1 cardiac and gastrointestinal patient (patient number 25) presented lower absorbance levels with anti-*T*. *cruzi* than the cut-off values (Fig 2B and 2C).

Two cardiac patients (patients number 16 and 18), 3 gastrointestinal patients (patients number 43, 45 and 46) and 2 cardiac and gastrointestinal patients (patients number 25 and 27) had lower absorbance levels with anti-MASP SP than the cut-off values (Fig 2B and 2C).

The results of antigen recognition in all patient groups, (cardiac, gastrointestinal and cardiac plus gastrointestinal pathologies) shows that the detection of EVs was more effective with

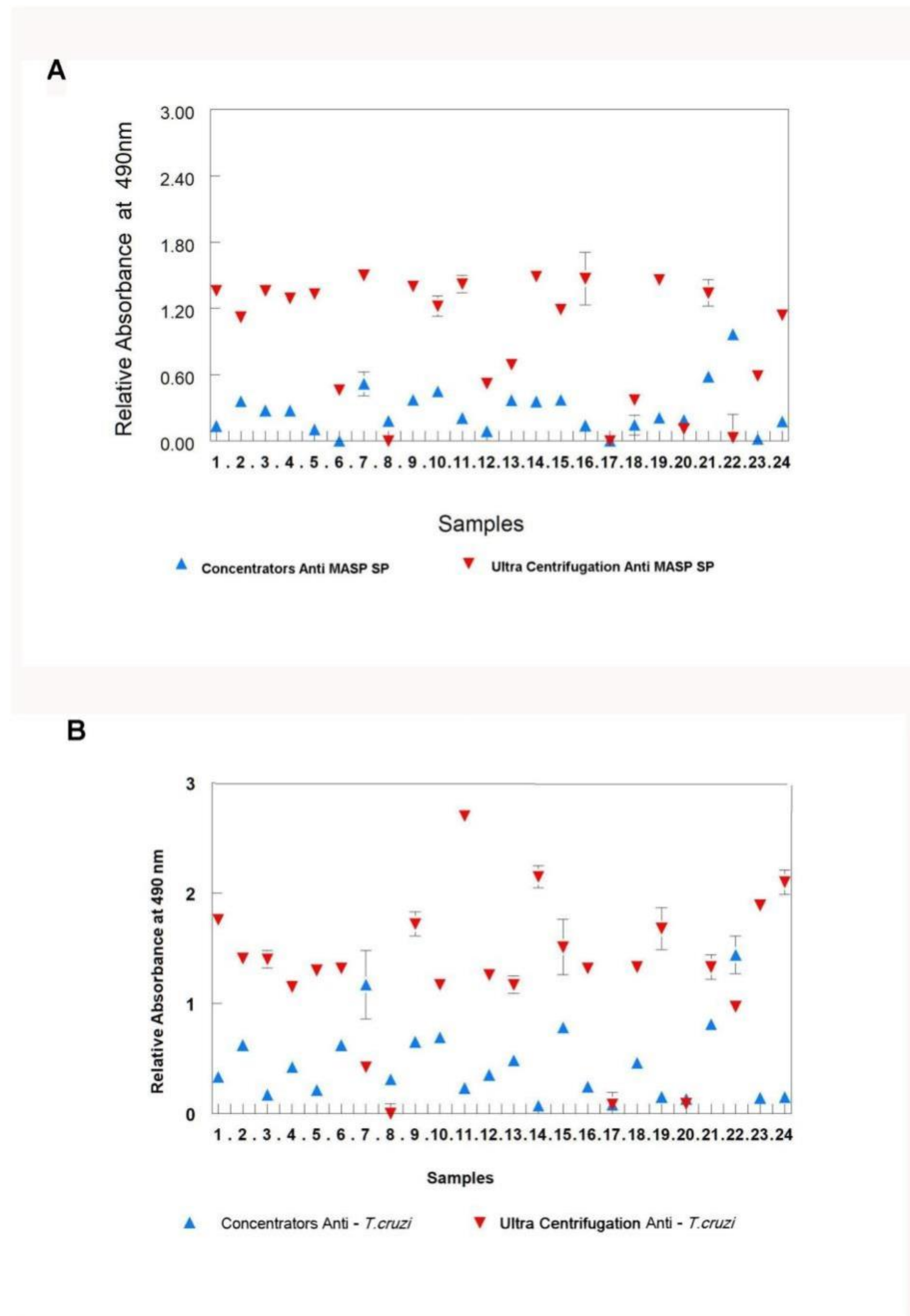


**Fig 1. Antigenic recognition of EVs obtained from sera of patients with CCD by concentrators vs differential centrifugation.** Relative absorbance was calculated as the absorbance values at 490 nm, minus the mean absorbance value of the cut-off value, obtained by the same immunoassays and treatment against EV from individuals without CD. A. Developed with anti-MASP SP immunoserum. B. Developed with anti-*T. cruzi* immunoserum.

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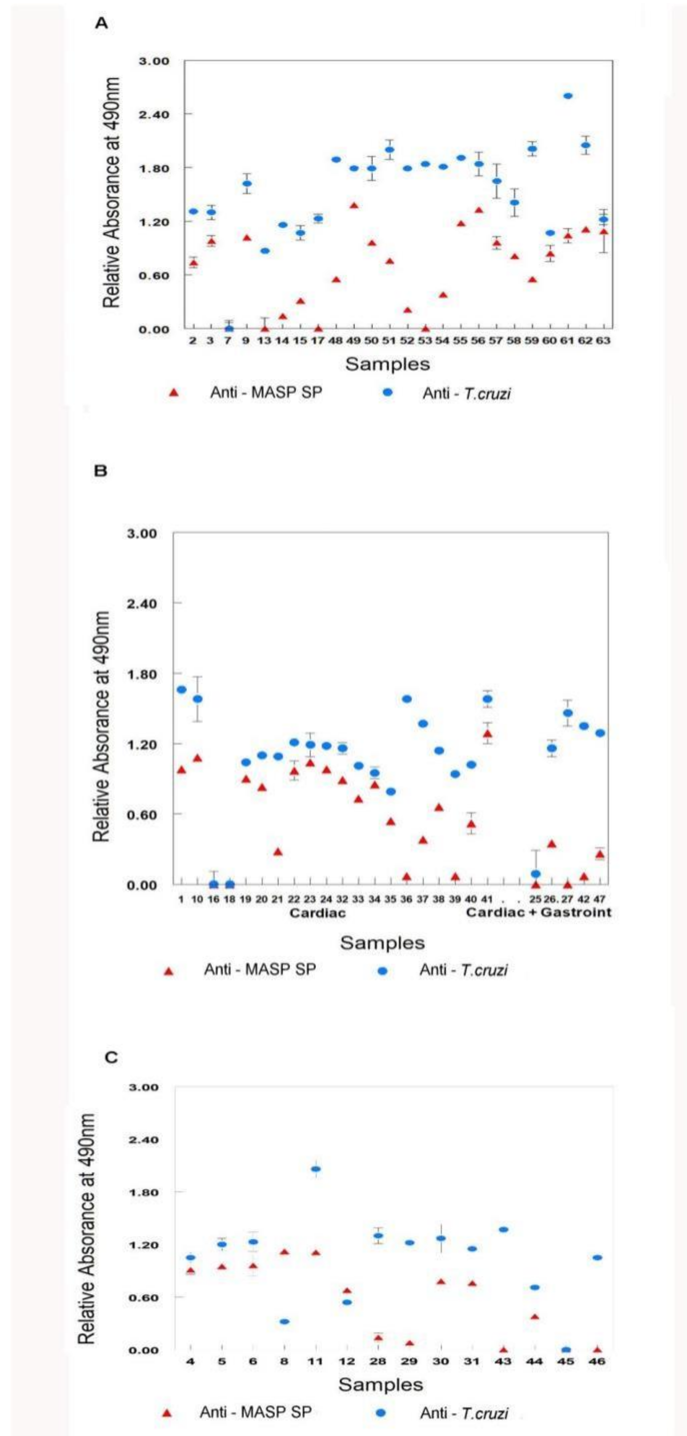


**Fig 2. Antigenic recognition by anti-*T. cruzi* and anti-MASP SP immunosera of EVs obtained by ultracentrifugation from sera of Bolivian patients with CD diagnosed in Spain and classified by symtoms.** The absorbance value at 490 nm is represented on the y-axis and samples from different patients are represented on the x-axis. The absorbance results are the net values after subtracting the cut-off value from the total absorbance A. Patients with CD and indetermined symthoms. B. Patients with CD and cardiac disorder, and with cardiac and gastrointestinal symptoms together. C. Patients with gastrointestinal symptoms.

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**Table 2. A summary of the results obtained with anti-*T. cruzi* and anti-MASP SP in the different clinical situations.**

| Patients | Total number | anti-*T. cruzi* | | | anti-MASP SP | | |
|---|---|---|---|---|---|---|---|
| | | % Positivity | Mean | Mediana | % Positivity | Mean | Mediana |
| **Indeterminates** | 24 | 95.8 | 1.83 | 1.9 | 83 | 0.83 | 0.93 |
| **Cardiac** | 20 | 90 | 1.21 | 1.16 | 90 | 0.72 | 0.82 |
| **Gastrointestinal** | 14 | 92 | 1.19 | 1.28 | 78 | 0.66 | 0.79 |
| **Cardiac and Gastrointestinal** | 5 | 80 | 1.31 | 1.38 | 60 | 0.21 | 0.13 |

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anti-*T. cruzi* immunoserum than with anti-MASP SP (Mann-Whitney U 993.5, $p < 0.00001$) for all the patients with different disorders.

When we evaluated the antigenic recognition obtained in EVs using anti-*T. cruzi*, significant differences were observed in the absorbances obtained depending on the pathology shown by the patients (Kruskal-Wallis test, chi-square = 14.536, df = 3, p-value = 0.00226). The group of patients with indeterminate symptoms showed significantly higher absorbance values compared to the absorbance obtained with circulating EVs from the cardiac and gastrointestinal patient groups (Bonferroni-corrected significance level method, p-value $<$0.05). However, this trend was not statistically significant when the anti-MASP SP immunoserum was used (Kruskal-Wallis test, chi-square = 6.1322, df = 3, p-value = 0.1054).

## Sample selection. Patients in Endemic areas, Panama

The rapid tests applied allowed us to obtain quick and reliable results, capable of having a first sweep of the population under study, mainly in the rural area where health resources are scarce, since their results are qualitative or semiquantitative and the samples do not require any type of equipment, testing system or specialized refrigeration. In the rural patients of the Charare´ community, 87.8% (29/33) were positive, while 75% (15/20) of the urban patients analyzed presented positive results in the rapid test (S1 Table).

A second commercial ELISA test (Wiener lab) was conducted in 53 chronic Chagas patients. Among them, 54% (29/53) tested positive, while 6% (3/53) were classified as clearly inconclusive due to their absorbance values falling within the "grey" zone or at the cut-off value (cut-off = 0.3). Additionally, 40% (21/53) tested negative. Serum samples from a rural population displayed a 42% (14/33) positivity rate for the aforementioned ELISA test method, while sera from urban patients selected from the hospital showed a 75% (15/20) positivity rate. All serum samples were evaluated in triplicate, and the results were recorded (Fig 3 and Table 3).

The WB analysis reveals distinct antigenic bands in positive sera (25, 30, 45, 52, 70 kDa), while the remaining bands are regarded as nonspecific for CD diagnosis. S4A Fig illustrates results from patients in urban areas, where 90% (18/20) tested positive, and 10% (2/20) yielded inconclusive results. In contrast, patients from the rural community of Charare´ (S4B Fig), exhibited an 84% (28/33) positivity rate, with 15% (5/33) showing indeterminate results (Table 3).

## Detection of circulating parasite EVs from the sera of CCD patients from an endemic country (Panama) by anti-*T. cruzi* immunoserum. proof of concept

As proof of concept to evaluate the utility of using circulating EVs as an indicator of the active presence of the parasite in sera where immunological diagnostic systems had been

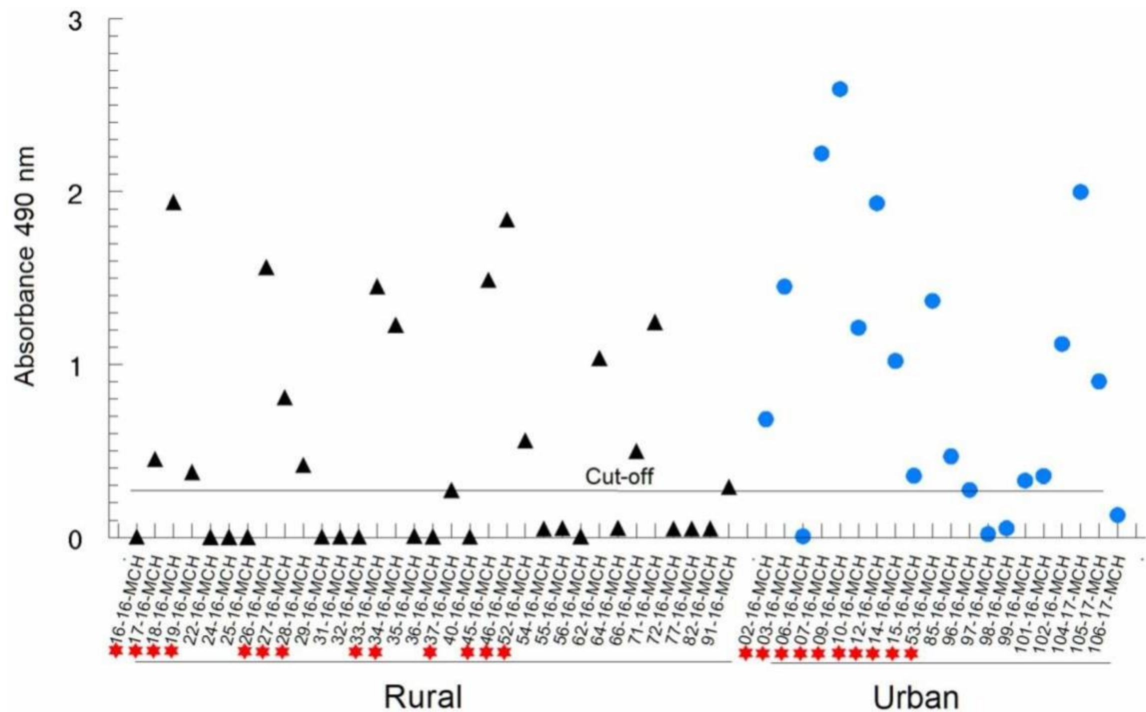


**Fig 3. Absorbance obtained by ELISA with the different serum samples. Comparison between rural and urban samples of patients diagnosed with CD in the Panamanian population.** Black triangles for patients living in rural areas and blue circles for urban patients in Panama City. Red asterisks show sera that were selected for proof-of-concept for Fig 4 used in subsequent experiments with antigen recognition in circulating EVs. The horizontal line represents the cut-off value of the negative sera obtained from the mean of the absorbances of these sera plus three times the standard deviation of the means of these negative sera (mean absorbance of negative sera +3 x SD).

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inconclusive, we decided to purify EVs from a total of 25 (16 positive for all techniques; 2 negative for the rapid test; 2 negative for the Wiener ELISA test; 2 negative for WB and one negative for WB and ELISA) as indicated in the text and caption of Fig 4 including a positive control and a negative control. This includes all diagnostic situations of these patient sera of Central American CCD patients from Panama that were in such a situation despite testing PCR positive. Once the circulating immunocomplexes from the serum were purified and any accompanying IgGs that could form immunocomplexes were eliminated, these EVs were confronted with anti-*T. cruzi* immunoserum. Negative controls consisted of EVs extracted from panel of 50 serum samples from immunologically negative individuals who lacked symptomatology after medical evaluation of these individual sera from the same geographical region as described in S3 Table and above, but lacking the disease, and treated with a similar procedure as the sera under study.

**Table 3. A summary of the results obtained with commercial tests and our proof of concept test.**

| Test % | Positive | Negative | Inconclusive |
|---|---|---|---|
| **Rapid test** | 83 | 17 | 0 |
| **Wiener ELISA** | 54 | 40 | 6 |
| **WB** | 90 | 0 | 10 |
| **anti-T. cruzi ELISA** | 100 | 0 | 0 |

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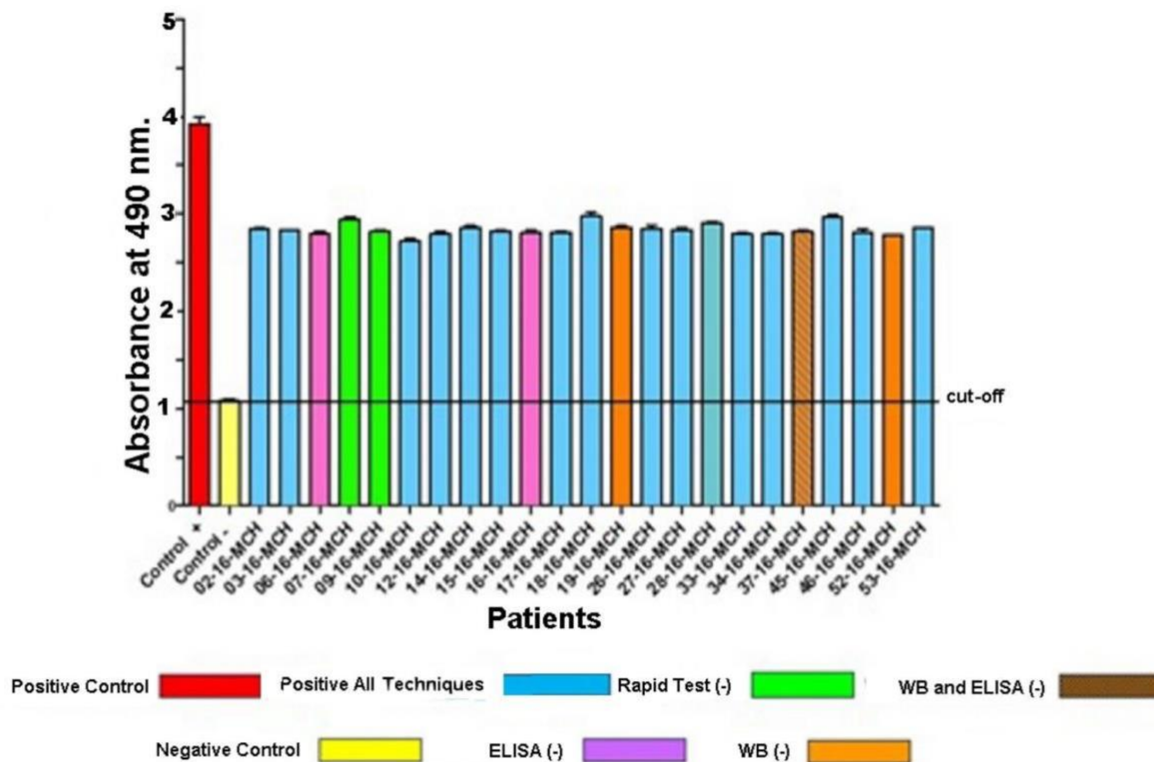


**Fig 4. Detection by ELISA of EVs with *T. cruzi* antigens in the serum of patients whose diagnosis by conventional diagnostic systems was inconclusive.** Red: positive control; yellow: negative control; blue: patients with CCD positive by PCR; Green: patients with CD but negative by rapid test; Brown: patients with CD and negative by WB and Wiener ELISA test; Purple: patients with CD negative by Wiener ELISA test; Orange: patients with CD positive by rapid test and Wiener ELISA but negative by WB.

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Fig 4 shows that anti-*T. cruzi* antisera recognize antigens in EVs isolated from the sera of 23 patients from Panama who presented inconclusive or negative in commercial diagnostic tests for CD, including WB results. In all cases, the absorbance obtained was higher than the cut-off value obtained from the pool of sera from non-infected individuals.

## Detection of circulating parasite EVs in sera of pregnant Bolivian Women and their infants

The study on the presence of EVs in pregnant women who tested positive for CD and sequentially tested for reactivity of EVs in their babies at 1 month and subsequently at 9 months after birth revealed that the mean absorbance value at 490 nm for anti-*T. cruzi* in the babies at the first month was 2.08 (95% CI: 1.77–2.27), whereas at 9 months, the obtained global result was 1.24 (95% CI, 0.74–1.61). Furthermore, a significant correlation was found with the Spearman test between absorbance values at 1 month and 9 months (Rho 0.72; p-value = 0.009) (Fig 5).

In samples corresponding to children number 5 and 13, the absorbance values were equal to or below the cut-off value using the immunoserum against total *T. cruzi* antigen at nine months after birth (Fig 5).

Samples from patients number 7, 9, 11 and 12 at 9 months of age could not be collected as they did not return to the hospital for follow-up of the mother or child.

Sample number 16 corresponds to a child who received treatment two months after birth due to the positive PCR of the mother during pregnancy and subsequently of the child one

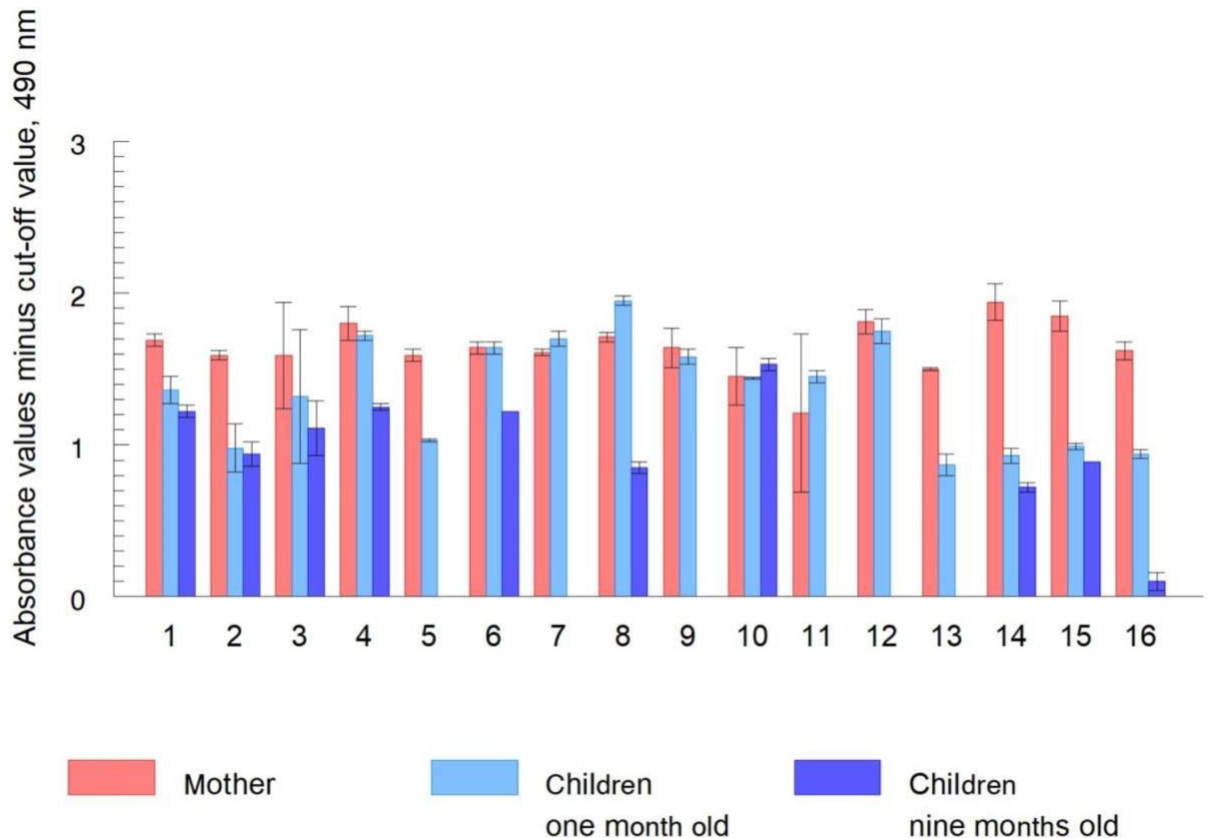


**Fig 5. *Trypanosoma cruzi* antigenic recognition in EVs obtained from sera of mothers with CD and their children by anti-*T. cruzi* immunoserum.** The absorbance value at 490 nm is represented on the y-axis and samples from 16 different mothers and their children are represented on the x-axis. The absorbance results are the net values of subtracting the absorbance of the cut-off value from the absorbance obtained from the samples.

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month after birth. The treatment consisted of Benznidazole, administered orally in three doses for 60 days, 10 mg/kg per day for the child. The mother received the same drug administered orally in two doses for 60 days at 5–7.5 mg/kg per day. At the beginning of the treatment, lactation was interrupted. The absorbance after recognition of the EVs by the anti-*T. cruzi* immunosorbent serum at 9 months after birth was very low in this child, treated and with interrupted breastfeeding.

Children in samples 5 and 13 showed significantly lower absorbance levels (similar or lower than the cut-off value at 9 months) between samples taken at 1 and 9 months of age in contrast to samples from children who still showed elevated absorbance at 9 months, (1, 2, 3, 4, 6, 8) although they experienced lower absorbance values compared to those obtained from mothers or at 1 month after birth, except for the child in sample 8, where the absorbance at 1 month was higher than that of the mother. The HSD Tukey test revealed a p-value $<0.001$ (Fig 5).

In sample 10, the values of circulating antigens in the parasite EVs, were maintained from the first month to the ninth month (HSD Tukey test revealed a p-value $>0.05$), while in the rest of the samples the absorbance values decreased with respect to those obtained at one month after birth.

The samples from mothers 1, 2, 14, and 16 tested positives in the PCR. While mothers 2, 7, 9, 11, 14, 15 and 16 were treated, the children of these mothers tested negative in the PCR at 2

months after birth. Except for number 16, who tested positive in the PCR and received treatment as indicated above (S2 Table). S1 Table shows the peculiarities of each of the cases in detail.

### Study of IgG subclasses in the immunocomplexes from Chronic CD patients (Bolivia and Panama) by ELISA

After purifying circulating immunocomplexes (IgGs-EVs) from the serum of CD patients using the ultracentrifugation method described above, we isolated the IgG antibodies that are part of the immunocomplexes to characterize the subclasses of IgGs forming them.

The results of the Bolivian patients in Spain are represented in Fig 6A. In this analysis, significant differences were observed in the absorbance values between the different IgG subclasses when compared with the different pathologies (ANOVA, Isotypes: p-value $<0.0001$; Pathology: p-value $<0.0001$). Specifically, IgG2 and IgG4 isotypes exhibited statistically higher levels (Tukey HSD test with 95% CI: diff = 12.11, p-value = 0 and diff = 9.36, p-value = 0, respectively), while IgG3 did not show significant differences compared to the other subclasses (Tukey HSD test, p-value $>0.05$).

When comparing Bolivian patient groups categorized as indeterminate and symptomatic (including cardiac, gastrointestinal, and combined cardiac plus gastrointestinal pathologies) via ANOVA (p-value $<0.05$), significant differences were found for each isotype. In all cases, the indeterminate patient group exhibited significantly higher levels compared to the diagnosed and pathologically affected patient group, as indicated by the following statistics IgG1 (Tukey test with 95% CI: diff = -1.946763, p-value $>0.0001$); IgG2 (Tukey test with 95% CI: diff = -6.996506, p-value $>0.0001$); IgG3 (Tukey test with 95% CI: diff = -1.387083, p-value $>0.01$) and IgG4 (Tukey test with 95% CI: diff = -4.088365, p-value $>0.001$).

All the patients from Panama graphed in Fig 6B were diagnosed with cardiac conditions. Interestingly, significant differences were found between all variables (IgG1, IgG2, IgG3, and IgG4) when compared to each other, following adjustment for multiple comparisons using the ANOVA method (p-value $<2e\text{-}16$ ***). The mean values of the IgG2 and IgG4 isotypes were significantly higher than those of IgG1 and IgG3 (Tukey HSD test, p-value $>0.05$). However, the absorbance values of IgG2 and IgG4 were found to be similar for these patients (Tukey HSD test, p-value $>0.05$).

## Discussion

The search for specific biomarkers for the diagnosis and prognosis of Chagas disease (CD) continues to be a research challenge to identify the presence of the parasite and the status as well as the prognosis of the development of the disease and that are capable of determining the response to treatment [1,56–60].

While in the acute phase the diagnosis with parasitological techniques confirms *T. cruzi* parasitism, the disappearance of the flagellate forms from the bloodstream together with the sustained presence of anti-*T. cruzi* antibodies throughout life during CCD constitutes a drawback for a serological evaluation to study the effectiveness of treatments [57], the risk assessment in blood or organ donors or the confirmation of infection in neonates.

In addition, the variability of the results of immunological tests based on the detection of antibodies, where the same patient may present disparity of results depending on the type of test to which he/she is submitted [61], a consequence of possible cross-reactions, the diversity of antigens due to the genetic variability of the parasite [62] and even the geographical differences of the parasite strains used [16,19,63] represents a challenge in CCD diagnosis. All this means that there are national and international recommendations, PHAO or WHO, on the

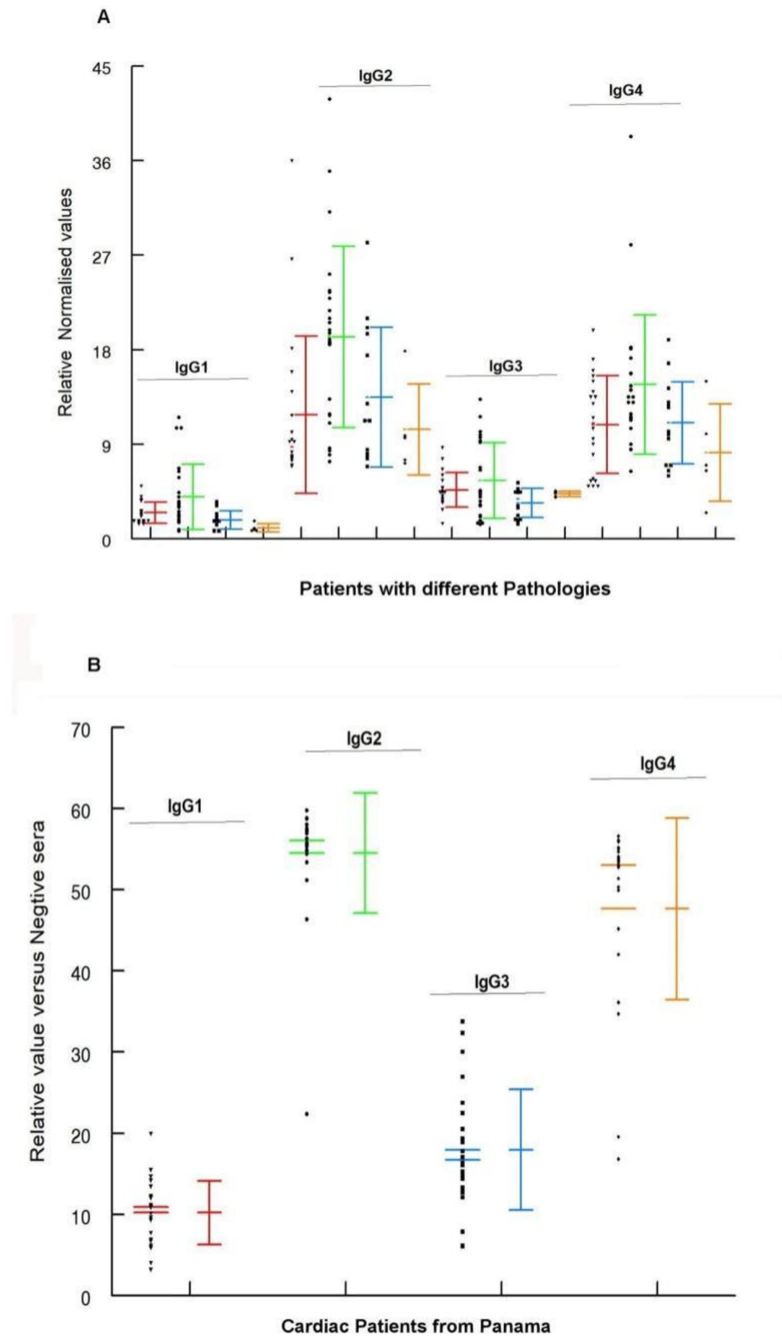


**Fig 6. Boxplots illustrating the relative absorbance values measured at 490 nm for each IgG subclass isolated from immunocomplexes obtained from patients with CD.** A. Mean values of patients originating from Bolivia with different pathologies. Red: cardiac pathology; Green: indeterminate patients; Blue: gastrointestinal pathology; Orange: combined cardiac plus gastrointestinal symptoms. B. Patients with CD originating from Panama. Mean and SD are represented for each isotype. Red: IgG1; Green: IgG2; Blue: IgG3 and Orange; IgG4.

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need to perform several immunological assays and that there should be no discrepancies in the positivity of these assays before diagnosing patients as positive [2].

Moreover, standardisation of methods to demonstrate the metabolically active presence of the parasite in affected individuals is necessary in both experimental and clinical evaluation of treatment efficacy. This also applies to the confirmation of parasitism in newborns born to mothers with the disease, and where the earliest possible treatment will allow these children to be cured [58,64] or in cases of follow-up treatment [65,66] as well as in cases of organ and tissue transplant donors who confirm the absence of the parasite in donors from endemic regions, or who have spent significant time in these areas [67,68].

Among the methods that denote the active presence of the parasite in patients, especially designed to detect blood trypomastigote forms in the acute phase of the disease, are the visualization of blood smears stained with Giemsa, or biological enrichment techniques for those cases with low parasitemia; xenodiagnosis with the vector or direct inoculation of blood to laboratory animals, are not applicable in diagnostic laboratories especially in regions where vectorial transmission does not take place. Other techniques based on the isolation and purification of blood trypomatigote forms such as Strout's technique [69] are easy to develop, but this particular technique requires a high blood volume of about 10 ml, from which, by means of two centrifugations, the blood protozoa can be concentrated and subsequently visualized microscopically. A variant where the volume of the blood sample is significantly reduced to 0.3–0.6 ml and applicable both in the acute phase and in studies of vertical transmission to neonates is the method developed by Freilij 1983 or microhematocrit concentration method [11,64,70]. All these techniques are limited to concentrate and visualize by buffy coat the presence of blood parasites, potentially infecting forms that for handling, are subject in the EU and USA to biosafety standards for handling level 3 in the EU and 2 in the USA [71] which implies avoiding the use of glass or metal and sharp material, which precludes the use of glass microcapillaries to carry them out. On the other hand, these techniques would only detect parasitemia above 50 tryp/ml) and not the presence of parasitism in cells and tissues with quiescent amastigotes and the absence of blood forms, which often cause therapeutic failure [72,73].

In other biological models such as quiescent muscle cells [74] secretion of EVs by such cells could be ascertained, suggesting the possibility of production of EVs released by such amastigotes forms.

A recent review of diagnostic techniques was compiled by Schijman A et al 2024 [75]. Among the most recommended serological methods to confirm Chagas disease are those that use trypomastigote excreted-secreted antigens (TESA) to detect antibodies from the patient, which react with proteins or glycoconjugates released by *T. cruzi*, thus consisting of the response to the set of excretion products of the trypomastigote forms of the parasite [76–80].

Although serological assays (ELISA and immunoblot) using TESA are very sensitive, they are still tests that evaluate the titers of antibodies against parasite antigens with the existence of cross-reactivity for patients infected by *Leishmania* ssp [81,82].

Proteomic studies of the TESA antigen reveal the presence of proteins already described in other analyses of the proteome of EVs and particularly Transialidases, GP63, highlighting the presence of retrotransposon hot spot proteins (RHS), that Bautista-Lopez et. (2017) [81] identified and characterised for use as diagnostic markers. In a previous study carried out in our group [48], it was found that the exosomes of trypomastigote forms derived from cell culture showed that 22% of the total protein types corresponded to Transialidases belonging to the groups I-VIII proposed by Freitas et al. (2011) [83] and Nardy et al. (2016) [84], these enzymes being found on the surface of the EVs [49] carrying in turn the cysteine protease Cruzipain and the non-orthologous metalloprotease GP63, likely to be responsible for the cross-reactions observed when TESA is used as a diagnostic antigen, since it is found in both *Leishmania* ssp

and *T. brucei* [85,86]. Subsequently Nagarkatti et al. (2020) [87] developed an antibody against a sequence of the Tc_517 peptide present in the *T. cruzi* secretome whose presence they use as a biomarker in serum for *T. cruzi* infection.

The use and usefulness of EVs in the diagnosis of various diseases including infectious diseases such as latent tuberculosis has been published recently [88–90]. Immunocomplex cargoes in patients with CD include Transialidase proteins or GP63, proteins characteristic of trypanosomatids [24]. When these immunomplexes were sequenced, the presence of Transialidase proteins or GP63, proteins characteristic of trypanosomatids, was found. The presence of circulating immunocomplexes consisting of parasite EVs and antibodies against the parasite in the circulating blood of chronic CD patients was described by D´ıaz-Lozano et al. (2017) [29].

Analysis of circulating immunocomplexes found in the serum of chronically ill patients revealed recognition of the signal peptide (SP) by immunogold techniques under MASP SP electron microscopy in 45.19% of the EVs forming these immunocomplexes, and by immunohistochemistry a maximum absorbance in immunocomplexes from chronically ill patients with digestive pathology [29].

The diagnostic use of PCR and real time qPCR using these immunocomplexes from patients with chronic Chagas disease was recently published by Lozano et al. (2023) [52], demonstrating that these circulating immunocomplexes carry and preserve the DNA from the parasite nucleus as well as from the kinetoplast (KDNA) of mitochondrial origin of the protozoan. EVs are capable of carrying and preserving both DNA and RNA of cellular origin [54,91].

In the present work and as a proof of concept, immunocomplexes with the EVs of the parasite in the serum of chronic Chagas disease patients were isolated. For their isolation and purification, the purification of EVs by ultracentrifugation (with a diameter of 209 nm with a mode of 166 nm) was compared with another methodologically and economically simple method, based on the use of single-use protein separators/concentrators (with a mean particle diameter of 240 nm with a mode of 208 nm). With the latter method, the concentration of total proteins decreased slightly with respect to those purified by ultracentrifugation, perhaps due to the absorption of liquids with dissolved proteins by the matrix of the filtration equipment (S2 Fig). These protein concentrating filters have already been used in the purification of EVs in different biological fluids or culture media [54,92–94].

Our results indicate that the choice of technique depends on our specific objective and the technological facilities of the laboratory to isolate circulating EVs in serum and determine the presence of biological material of the active forms of the parasite.

That is, if our objective is to obtain a higher signal provided by circulating EVs, the method of choice would be ultracentrifugation, since a higher amount of protein is obtained in these EVs.

In order to check the presence of the IgG subclasses that form them in the immunocomplexes purified after dissociating the immunoglobulins as described in Material and Methods, these were characterised by ELISA. The results are shown in Fig 6A and 6B, where it can be seen that, in both the Bolivian and the Panamanian patients, IgG2 and IgG4 were the highest titres of the IgGs forming the immunocomplex. The determination of immunoglobulins in Chagas disease has been studied by several authors. Brodskyn et al. (1989) [95] studied IgGs in Chagas disease, suggesting that the immune clearance of *T. cruzi* is due to antibodies located in the IgG isotype, particularly in the IgG2 subclass. Similarly, in an experimental study carried out by Spinella et al. (1989) [83], the main IgG subclass found was IgG2a, reaching 10 times the control level especially in the chronic phase of the disease. This would indicate that some of the EV antigens, possibly glycosylated, would stimulate the antibody response and must be recognised by IgG2.

The lower recognition by the anti-MASP SP could be explained by the data obtained by D´ıaz-Lozano et al. (2017) [29] where it was observed that only 45.19% of the EVs isolated from the trypomastigote forms presented gold tags when performing immunochemistry under TEM and the number of tags per EV was 1.41 ± 0.65, i.e., only approximately half of these EVs carried the recognised epitopes of the highly specific peptide of *T*. *cruzi* and belonging to what could be considered immature MASP proteins. However, in the proteome of EVs from trypomastigote forms there are 524 proteins, of which 250 are specific for trypomastigote forms [48]. Therefore, the chances of the epitopes of these proteins being recognised is significantly higher than those present in the signal peptide of the MASP proteins.

As proof of concept of the use of circulating EVs in serum forming immunocomplexes, as an indicator of the presence of material from metabolically active parasites, and based on the results obtained with the different diagnostic tests in the Panamanian patients: i) sera positive for all three diagnostic tests; ii) with those where the immunological diagnosis was negative or inconclusive with the different methods tested, including the rapid tests, the Wiener ELISA test (Fig 3) or the WB (S4 Fig), tests with circulating EVs would be recommended to confirm active parasitization.

As indicated above, and in order to test in a series of patients in whom the different traditional diagnostic methods were not conclusive, a total of 23 individualized sera were selected in which positivity had already been proven by PCR performed at the University of Granada in whole blood samples transported with guanidine, in which even DTUs causing the infection were determined [50]. From parallel samples of sera from these patients, circulating exovesicules were extracted by ultracentrifugation. In that panel of sera, there were seven in the above conditions (negative with any of the three techniques or inconclusive). As can be seen in the graph in Fig 4, the absorbances obtained when confronted with EVs to the anti-*T*. *cruzi* immunoserum are shown, all the selected sera gave absorbance values higher than the cut-off value obtained with EVs from the negative control subjects. This demonstrates the presence of metabolically active parasites in all the cases analyzed.

Congenital Chagas disease has now acquired epidemiological relevance, especially after the insect vector control campaigns carried out in many endemic countries [96], and currently remains a crucial challenge for both endemic and especially nonendemic countries, where this form of transmission, along with transfusion or transplantation, would be the only way of spreading the disease in these countries far from vector transmission [97–99]. However, due to the neglected nature of the disease and persistent barriers to access diagnosis, treatment and care, the prevalence in pregnant women and their newborns may be underestimated [10,64,100].

An estimated 1.12 million women of childbearing age are infected by the *T*. *cruzi* parasite [56,64], where the prevalence of vertical transmission approaches 5% [101]. The incidence of congenital Chagas disease is estimated to be between 8,000 and 15,000 cases per year in Latin America [64].

Of the Bolivian patient population assessed in this study, 16 were pregnant women (S2 Table) who tested positive for Chagas disease. Four of them had been diagnosed by PCR prior to pregnancy and seven of them treated with Benzinidazole, (the standard treatment prescribed was Benznidazole, administered orally in three doses for 60 days at 7.5 mg/kg per day for mothers and 10 mg/kg per day orally in children, who tolerated the treatment better). although numbers 14, and 16 did not undergo treatment control as some of them were PCR positive again when they became pregnant, had circulating immunocomplexes isolated from the serum of their infants at one month after birth and at 9 months (S1 Table). It is noteworthy that the umbilical cord blood was not available in any of these cases at the time of birth, as would have been desirable. In the days prior to delivery, some patients (1, 2, 14 and 16) were

PCR positive, while only the son of mother 16 was PCR positive and was treated together with his mother, starting two months after birth, for which he was withdrawn from breastfeeding.

The results obtained from PCR in newborns are considered inconclusive due to the limited amount of blood collected, and DNA purification, carried out at the hospital, by automated DNA purification methods [52].

In all cases, the absorbance obtained with the vesicles purified from the sera from both mothers and offspring gave higher values than the cut-off value obtained with EVs from individuals negative for the disease.

Of note is the decrease in absorbance in the infants at one month after birth with respect to that obtained in the mothers, except in cases 7 and 8. and as already indicated, samples were not available from the infant of patient 7, since the mother did not return with him to the nine-month follow-up.

In the case of baby number 10, the absorbance at one month was maintained with respect to that obtained in his mother, increasing the absorbance at 9 months with respect to the values obtained from the other two samples, including the mother's sample. Although at one month and later he was PCR negative, in a medical check-up one year after birth and before returning to his country of origin, he tested positive in the PCR test and treatment was recommended. Therefore, it could be considered as a false negative for PCR, due to the problems mentioned above, shortage of blood for analysis or shortage of purified DNA.

Sample 16 was different, i.e., both mother and baby tested positive for PCR in the study performed at one month after birth. Both were subjected to treatment two months after birth and breastfeeding was interrupted. In this case, and perhaps as a consequence of the treatment, the values of parasite EVs decreased at 9 months, with the result that the abbsorbances decreased considerably until they were slightly higher than the cut-off value. It should be remembered that the values represented on the ordinate axis are the values of the mean absorbance of the sample, minus the mean value of the cut-off.

This decrease in absorbance could be considered indicative of the efficacy of the treatment, by decreasing the active forms of the parasite and thus the release of circulating *T*. *cruzi* EVs recognized by the anti-*T*. *cruzi* immune serum.

During normal pregnancy, the presence of EVs in the fetal circulatory system and communication between the mother and the growing fetus occur through the exchange of EVs produced by both the mother and the fetus [102,103]. Exosomes derived from the placenta may represent a mechanism by which the placenta communicates to induce maternal adaptations to pregnancy, and these EVs may serve as potential markers for various fetal and maternal pathologies during pregnancy [104–106]. EVs, like those obtained from umbilical cord blood, neonatal blood, or even urine, serve as markers for neonatal pathologies, particularly in prematurity and during the perinatal adaptation period, from birth until approximately 4 weeks after delivery [107].

In our case, as we did not have access to and analyze umbilical cord blood [108], we must assume that the EVs forming immunocomplexes found in the blood of newborns do not originate from the exchange of mother-fetus EVs through the placenta. This assumption is based on the short half-life of EVs in the circulatory system, as estimated in experimental studies [108]. The first sample analyzed from the blood of the infants was taken one month after birth during the first CD screening, as these infants were born to mothers with a history of infection. Therefore, the EVs detected in the infants' serum, identifiable by antibodies from anti-*T*. *cruzi* immune serum, either originate directly from the infected children due to transplacental infection or may have maternal origin through breastfeeding.

The presence of EVs in colostrum [109] and breast milk has been associated with infant development [110]. Exosomes derived from breast milk have functions related to the maturation of the immune system [111], contributing to the increase in the number of regulatory T cells in peripheral blood, possibly to regulate immune tolerance [112]. It has also been demonstrated that exosomes derived from breast milk promote the proliferation of intestinal epithelial cells [113].

On the other hand, maternal immunoglobulins, primarily IgGs and IgA, present in colostrum and breast milk [114], contribute to maintaining immunity during infancy, as well as tolerance to intestinal bacterial flora and even the transfer of vaccine-induced antibodies to protect both the mother and the child from infectious diseases [115,116]. The passage of intact IgGs into the newborn's circulatory system, after being ingested with breast milk, occurs through the intestine with the involvement of FcRN receptors to which IgGs bind [117,118]. The receptor binds immunoglobulin G (IgG) and albumin, retrieving them from degradation and transporting these ligands through polarized cellular barriers via a pH-dependent binding and release mechanism. These processes ensure the distribution and high levels of IgG and albumin throughout the body. These receptors are present in mucous membranes and particularly in the polarized cells of the intestinal wall [117,119], which facilitate the passage of IgGs from the intestinal mucosa to the newborn through transcytosis [120,121]. Monomeric IgGs and IgG immunocomplexes can be transported from either the apical or basolateral side of mucosal cells, where these receptors are located, to acidic pH endosomes, and the subsequent release of the receptor on the opposite cell surface in response to extracellular neutral pH. Different IgG immunocomplexes can pass through mucosal barriers via transcytosis [122], facilitating the mechanism of transcytosis for the passage of certain microorganisms forming immunocomplexes through mucous membranes and facilitating infection [123]. It is through this transcytosis mechanism that EVs containing *T*. *cruzi* antigens and forming immunocomplexes with IgGs could pass into the newborn's bloodstream when breastfeeding by mothers with a history of infection.

In the present study, the active presence of the parasite was detected in mothers through the recognition of circulating EVs containing parasite antigens by anti-*T*. *cruzi* serum. However, in newborns, a decrease in absorbance was observed at 9 months post-birth, including in case 16, where the decrease was more pronounced although both the mother and the baby had been diagnosed as positive by PCR at one-month post-birth and were undergoing treatment with benznidazole. Also, breastfeeding had ceased at two months post-birth, which could have contributed to the decline in recognition of circulating parasite antigens, in the form of immunocomplexes with parasitic material. In case 10, the increase in absorbances observed at 9 months post-birth could be attributed to a real *T*. *cruzi* infection not detected by the diagnostic methods used and later confirmed. In the remaining infants, the decrease in recognition of *T*. *cruzi* circulating exovesicle antigens may correlate with the diminished or lack in breast milk intake over time after birth.

## Conclusion

In conclusion, the ease, minimal equipment requirement, and low cost of isolating EVs forming immunocomplexes, primarily using serum protein concentrators, and *T*. *cruzi* antigen detection in EVs should be applied in cases where evidence of active parasite forms is required, both in patients in the chronic phase and in cases undergoing treatment. To ensure detection in newborns, it would be necessary to apply it to umbilical cord blood at birth or take precautions, i.e., to stop breast milk intake a few days before conducting the

test to ensure the parasitic origin of circulating immunocomplexes in the serum of these newborns.

## Supporting information

**S1 Fig. EVs quality and quantity control.** A. NTA results of the total EVs obtained from the sera by ultracentrifugation. B. NTA results of the sera EVs obtained by protein concentrators. C. Transmission electron microscopy of the sera EVs purified by filtration/ultracentrifugation. The arrows show the EVs. The measuring bar 500 nm. D. Transmission electron microscopy of the sera EVs purified by the protein concentrators. The arrows show the EVs. The measuring bar 200 nm.
(DOCX)

**S2 Fig. Graphic representation showing the protein load of sera EVs samples obtained by protein concentrators and vesicles purified by ultracentrifugation.** The red is EVs obtained by filtration with protein concentrators, while the blue represents the proteins obtained by ultracentrifugation. The blue line is the mean proteins of the samples obtained of the filtration procedure. The red line the mean of the samples obtained by filtration procedure. Each serum sample is represented on the x axis.
(DOCX)

**S3 Fig.** A. Electrophoresis in SDS PAGE of T. cruzi EVs and Immunocomplexes with EVs obtained from a pool of sera from cardiac patients. B. Antigenic recognition against MASP-SP by immunosera obtained against the synthetic peptide in the immunocomplexes obtained from patient sera. C. Antigen used in immunization against MASP-SP peptide. Four copies of the synthetic peptide were bound by branched Lysines.
(PDF)

**S4 Fig. WB analysis of antigenic recognition by sera from patients in Panama.** A. Results of patients from the urban area. B. Results of patients from the rural community studied. The WB analysis reveals distinct antigenic bands in positive sera (25, 30, 45, 52, 70 kDa).
(TIF)

**S1 Table. Data on the adults studied, age range, sex, origin, and diagnostic tests used and their results.**
(XLSX)

**S2 Table. Data on positive mothers and newborns.**
(XLSX)

**S3 Table. Data of the negative Controls used.**
(XLSX)

## Author Contributions

**Conceptualization:** Antonio Osuna.

**Data curation:** Mercedes Gomez-Samblas.

**Formal analysis:** Mercedes Gomez-Samblas, Antonio Osuna.

**Funding acquisition:** Luis Miguel de Pablos, Antonio Osuna.

**Investigation:** Noelia Lozano, Alexa Prescilla-Ledezma, Eva Calabuig, José Miguel Sahuquillo Arce, José Luis López Hontangas, Luis Miguel de Pablos, Mercedes Gomez-Samblas, Antonio Osuna.

**Methodology:** Noelia Lozano, Alexa Prescilla-Ledezma, Eva Calabuig, José Miguel Sahuquillo Arce, José Luis López Hontangas, Mercedes Gomez-Samblas, Antonio Osuna.

**Project administration:** Antonio Osuna.

**Supervision:** Maria Trelis, Antonio Osuna.

**Validation:** Eva Calabuig, Maria Trelis, José Luis López Hontangas, Luis Miguel de Pablos, Antonio Osuna.

**Visualization:** Mercedes Gomez-Samblas, Antonio Osuna.

**Writing – original draft:** Noelia Lozano, Alexa Prescilla-Ledezma, José Luis López Hontangas, Luis Miguel de Pablos, Mercedes Gomez-Samblas, Antonio Osuna.

**Writing – review & editing:** Eva Calabuig, Maria Trelis, Luis Miguel de Pablos, Mercedes Gomez-Samblas, Antonio Osuna.

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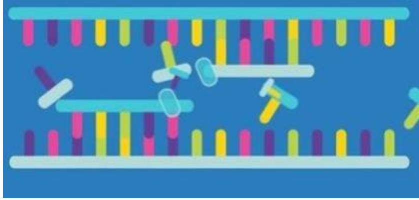

# *Use of sera cell free DNA (cfDNA) and exovesicle-DNA for the molecular diagnosis of chronic Chagas disease*

Noelia Lozano, Mercedes Gómez-Samblas, Eva Calabuig, María José Giménez Martí, María Dolores Gómez Ruiz, José Miguel Sahuquillo-Arce, Sergio Sequera- Arquelladas, José miguel Molina Moreno, María Trelis, Antonio Osuna.

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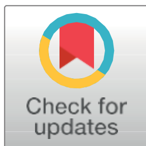
RESEARCH ARTICLE

# Use of sera cell free DNA (cfDNA) and exovesicle-DNA for the molecular diagnosis of chronic Chagas disease

**Noelia Lozano[1,2], Mercedes Gomez Samblas[3]*, Eva Calabuig[4], María José Giménez Martí[2], Maria Dolores Gómez Ruiz[2], José Miguel Sahuquillo Arce[2], Sergio Sequera-Arquelladas[5], José Miguel Molina Moreno[2], M. Trelis[1,6], Antonio Osuna[3]***

**1** Area of Parasitology, Department of Pharmacy and Pharmaceutical Technology and Parasitology, University of Valencia, Valencia, Spain, **2** Servicio de Microbiología y Parasitología Clínica, Hospital Universitario y Politécnico La Fe-IIS La Fe, Valencia, Spain, **3** Grupo de Bioquímica y Parasitología Molecular (CTS 183), Departamento de Parasitología, Campus de Fuentenueva, Instituto de Biotecnología, Universidad de Granada, Granada, Spain, **4** Unidad de Enfermedades Infecciosas, Servicio de Medicina Interna, Hospital Universitario y Politécnico La Fe-IIS La Fe, Valencia, Spain, **5** Unidad de Enfermedades Infecciosas, Hospital Universitario Virgen de las Nieves, Granada, Spain, **6** Joint Research Unit on Endocrinology, Nutrition and Clinical Dietetics, University of Valencia-Health Research Institute La Fe, Valencia, Spain

* aosuna@ugr.es (AO); msambla@ugr.es (MGS)



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## Abstract

Chagas disease, a neglected tropical disease, is now considered a worldwide health concern as a result of migratory movements from Central and South America to other regions that were considered free of the disease, and where the epidemiological risk is limited to transplacental transmission or blood or organ donations from infected persons. Parasite detection in chronically ill patients is restricted to serological tests that only determine infection by previous infection and not the presence of the parasite, especially in patients undergoing treatment evaluation or in newborns. We have evaluated the use of nucleic acids from both circulating exovesicles and cell-free DNA (cfDNA) from 50 samples twice randomly selected from a total of 448 serum samples from immunologically diagnosed patients in whom the presence of the parasite was confirmed by nested PCR on amplicons resulting from amplification with kinetoplastid DNA-specific primers 121F-122R. Six samples were randomly selected to quantify the limit of detection by qPCR in serum exovesicles. When the nucleic acids thus purified were assayed as a template and amplified with kinetoplastid DNA and nuclear satellite DNA primers, a 100% positivity rate was obtained for all positive samples assayed with kDNA-specific primers and 96% when SAT primers were used. However, isolation of cfDNA for *Trypanosoma cruzi* and amplification with SAT also showed 100% positivity. The results demonstrate that serum exovesicles contain DNA of mitochondrial and nuclear origin, which can be considered a mixed population of exovesicles of parasitic origin. The results obtained with serum samples prove that both cfDNA and Exovesicle DNA can be used to confirm parasitaemia in chronically ill patients or in samples where it is necessary to demonstrate the active presence of the parasite. The results confirm for the first time the existence of exovesicles of mitochondrial origin of the parasite in the serum of those affected by Chagas disease.

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## Introduction

*Trypanosoma cruzi* is a protozoan parasite etiologically responsible for American trypanosomiasis or Chagas disease (CD), a zoonotic disease among the so-called neglected tropical diseases. In addition to humans, the parasitism affects other mammals that can serve as reservoirs for the parasite. The insects involved in vector transmission are blood-sucking reduviids of the family Triatominae, with mammalian infection occurring as a result of contamination of the skin and mucous membranes with insect feces containing infective forms of the protozoan. Non-vectorial human infection can occur orally by ingestion of fruit juice contaminated with crushed insects or feces, by transfusion [1, 2] or by vertical transmission from mother to fetus [3]. These last two routes are epidemiologically important in areas where there is no vector transmission exists.

Chagas disease is endemic in a total of 21 countries in the Americas, from the southern United States to Argentina and Chile [4, 5], with approximately 10 million people infected and an annual incidence of 50,000 to 200,000 infections. For many years, the disease was considered confined to Mesoamerica and South America, but in recent decades it has spread to other parts of the world through migratory processes. Cases have been reported in the United States, Canada, European countries, Australia or Japan, areas with varying degrees of migration from endemic areas [6], making CD a public health problem in European countries such as Spain, Italy or Sweden, among others, where strict control measures have been adopted by blood banks [7], gynecological services [8] and transplant surgeries [9–11].

In the course of Chagas disease, there is an acute phase (ACD) that occurs in the first eight weeks immediately after infection. It is usually associated with a high number of circulating parasites in the blood, which facilitates both parasitological and molecular diagnosis, especially when patients present with pathognomonic symptoms, although the likelihood of this occurring is low. For this reason, most patients are referred to as being chronically ill without a diagnosis. The chronic phase (CCD), which develops 3 to 8 weeks after infection, may persist for decades either asymptomatically or with mild symptoms and very low levels of parasitemia. It is estimated that only 30% of cases develop disease-specific symptoms throughout the disease [12–14]. These symptoms include cardiac and/or gastrointestinal disturbances [15]. CCD poses the greatest epidemiologic risk of disease transmission in countries where infection by insect vectors does not occur, either as a consequence of blood transfusion or transplantation, or as a consequence of transplacental transmission from mother to fetus. Due to the low parasitemia in blood, detection of the parasite is very difficult and new techniques and/or molecular markers are being developed to demonstrate the presence of the parasites or secreted products that indicate their active presence [16–20].

Diagnostic methods vary from direct tests, such as xenodiagnosis, which may include a combination of molecular procedures [21], to immunological techniques using different antigens (native or recombinant) [22, 23]. The high variability in sensitivity and specificity between methods has been related not only to the type of laboratory technique or antigen used, but also to geographical differences in parasite strains, as well as genetic differences between human populations, which may contribute to discrepancies in serologic tests [24]. This has led to a series of recommendations from the Pan American Health Organization (PAHO) and national guidelines [25–27], which recommend the use of two serologic tests in parallel for confirmatory purposes, with a sensitivity of at least 98% [28] to correctly diagnose the disease. However, when serologic tests are inconclusive, some guidelines recommend molecular tests such as PCR, which is the technique of choice for the confirmatory diagnosis of *T. cruzi* parasitemia [29]. The WHO recommends PCR as a confirmatory test after blood donor selection, in the diagnosis of acute or congenital infection, or for therapeutic follow-up

after diagnosis of acute infection [28]. However, conventional PCR is not suitable for the diagnosis of chronic CD due to the low parasitemia in the chronic phase, which, together with the different extraction systems, blood sample preservation and DNA purification, makes PCR sensitivity around 50–90%, while its specificity remains close to 100% [30]. Due to the above-mentioned drawbacks, and despite its high specificity, PCR is only recommended when serologic tests are inconclusive, according to the recommendations of expert committees in different countries, such as Brazil, Chile, USA or Spain [25–27].

To address the challenges posed by patients with low parasitemia, such as the chronically ill or those requiring evaluation of treatment efficacy [31–33], new molecular methods have been developed over the last two decades. These methods aim to overcome the problems inherent in the disease itself or in the purification of DNA from blood samples. Thus, the use of chaotropic agents such as guanidine hydrochloride, which denature the proteins in the blood sample prior to DNA purification, new DNA extraction systems [17] and the use of specific primers that increase sensitivity [34, 35], especially for those hospital centers where the extraction of nucleic acids is carried out with automatic equipment and in which the extraction efficiency is not the most effective, this together with the development of PCR methodologies to increase sensitivity such as quantitative PCR (qPCR) [18] capable of detecting low concentrations of DNA (0.01 parasites in the sample), have gained ground. Also new amplification systems such as isothermal amplification (LAMP) [17, 36] that facilitate the implementation of molecular diagnosis are used. All of them aim to facilitate and increase the detection of the parasite in patients.

As in other diseases such as visceral Leishmaniasis [19, 20], in Chagas disease, the amount of circulating DNA of parasitic origin may be below the detection limits of molecular techniques. Therefore, new methods are sought to detect and demonstrate the active presence of the parasite. Among these methods is the detection in biological fluids of products of the particulate secretome, especially exovesicles of the parasite, where both proteins and nucleic acids make it possible to demonstrate the presence of metabolically active forms of the parasite [37].

Extracellular vesicles (EVs) are small membrane-coated vesicles released into the extracellular environment by almost any type of cell. EVs can be classified according to their size, biogenesis and composition; This classification includes: a) exosomes (20–100 nm), b) ectosomes (100–1000 nm) and c) apoptotic bodies (>1000 nm), among others [38, 39]. The composition of the EVs is complex and contains proteins, lipids, nucleic acids (DNA and RNA) [40, 41], including the EVs from *T. cruzi* [42]. EVs secretion by *T. cruzi* was first demonstrated by da Silveira et al., in 1979 [43] and, since then, several research groups have investigated the role of EVs in the pathogenesis of Chagas disease, demonstrating significant effects mainly on cell-cell communication, cell infection and evasion of the immune response [44, 45].

Although there are different methodologies both in the literature and commercially available, based on filtration systems, or through different chromatographic methods that facilitate the purification of the different exovesicles existing in biological fluids [41, 46–49] in the present work and as a proof of concept for the use of EVs in the molecular diagnosis of CD, we have followed the methodology considered the gold standard for the purification of exosomes, based on a mixed system of centrifugation/filtration/ultracentrifugation and the subsequent verification of the EVs purified by electron microscopy and nanoparticle tracking analysis (NTA), and which has been previously described by our work group [44, 50].

The presence of EVs of *T. cruzi* in the serum was previously demonstrated, and specifically, the presence of these EVs forming immunocomplexes containing specific *T. cruzi* proteins and without orthologues in other species in the serum of chronic patients with CD [50].

As a proof of concept, this paper demonstrates how the use of the parasite's cell-free DNA (cfDNA) as well as the EVs of the parasite's secretome present in the serum of chronic patients as "containers" that transport parasite DNA, can be used for molecular diagnosis of CD.

# Material and methods

## Human blood samples

Both, the blood and serum samples (n = 448) used in this study come from patients with symptoms compatible with CD or clinical suspicion and who attended the Hospital Universitario y Polite´cnico La Fe (HUyP-La Fe), Valencia, Spain during the years 2011–2020 where they were serologically analyzed with the LiaisonXL murex chemiluminescence kit and titrated with the Trinity Biotech IFA kit. Immunologically positive samples have been used in this study.

The samples included patients with suspected disease or with overt symptoms. These samples can be considered representative of the different population groups affected by CD, mostly adults (n 313), mostly born in Bolivia, with the exception of children born in Spain to mothers with Chagas disease (n 135). Some corresponded to patients who had received treatment for CD and who came for re-evaluation as follow-up, and patients with compromised immunity with post-transplant immunosuppression therapy; moreover, one of the patients was HIV seropositive. According to sex, 302 (67.4%) patients were female and 146 (32.6%) were male. All of the patients gave written informed consent before starting the study and all protocols were approved on March 4, 2011. The study was expanded to include the use to isolate and purify EVs from circulating serum and cell-free DNA (cfDNA) on September 26, 2018, with registration numbers 201102400000408 and 672/CEIH/2018 respectively, by the ethics committee of Granada University.

## DNA isolation method from peripheral blood

Peripheral blood samples (5 mL) were collected in Vacutainer vacuum blood tubes with EDTA and then subjected to a specific lysis pretreatment before proceeding to the DNA isolation methods. Pretreatment consisted of mixing 1:1 blood and denaturing lysis buffer (6M guanidine hydrochloride, 10 mM urea, 10 mM TRIS-HCL, 20% (v/v) Triton X-100; pH was adjusted to 4.4) through brief shaking. The samples were incubated at 70˚C for 10 min. Before proceeding to the next steps, the samples were kept for at least 48 h at room temperature.

The automated purification protocol of the Maxwell Blood DNA Purification kit (Promega Biotech) was chosen according to the guidelines approved by HUyP-La Fe and was used following the manufacturer's instructions. The initial volume for each of the blood sample was 400 μL of pretreated peripheral blood.

## Isolation of circulating cell-free parasite DNA (cfDNA) from serum

For the serum samples, 5 mL of blood were taken in the BD Vacutainer SST II Advance tubes (Reference. 366468). Once vortexed for 5 min, each tube was centrifuged at 1,500 x g for 10 min and approximately 2.0 mL of serum were collected to perform the different tests.

The samples used for the assay of this protocol were 4 serum pools and two individual serum samples from patients with digestive disorders. Two pools were formed by three patients with cardiac pathology each, and two pools formed by three patients with non-specific symptoms (S1 Table).

For the isolation of circulating DNA (cfDNA) of the parasite from the patient's serum, the MagMAXTM cell-free DNA isolation kit (Thermo Fisher Scientific) was used and the manufacturer's instructions were followed. First, the 100 μL serum pools were centrifuged at 1,600 x g 10 min at 4˚C. Once the first centrifugation was completed, the supernatant was subjected to a second centrifugation at the same speed and for the same time as the previous one in order to remove any cellular debris in the serum. Next, the proteins contained in the supernatant were digested for 20 min at 60˚C with 2 μL of Proteinase k (20 μg/mL). To the digestion

product, 150 μL of buffer (30mTris-HCl pH 8.0, 10mM EDTA, 1% SDS) were added together with 5 μL of magnetic bead solution. The resulting volume (255 μL) of the mixture was vortexed intensively for 10 min and then centrifuged at 14,000 x g for 10 s. The resulting pellet was washed twice, first with 500 μL of wash buffer and then with 500 μL of 80% ethanol, and subjected to centrifugation (20 s at 1,3000 x g). Finally, the pellet was dried and resuspended in 50 μL Milli-Q water.

## DNA isolation from serum exovesicles (EVs-DNA)

To improve the detection of parasite DNA in *T. cruzi* infected patients, DNA was isolated from exovesicles (EVs) present in serum. For this assay, 25 nested PCR negative and 25 nested PCR positive samples were selected as described below.

Purification of circulating EVs from serum was performed following the methods previously described by D´ıaz et al., 2017 [50] and Retana et al., 2019 [44], by a mixed ultracentrifugation-ultrafiltration procedure. For this purpose, 1 mL serum samples were each diluted (1:1) with PBS previously ultrafiltered through 0.22 μm pore filters. The diluted samples were first centrifuged at 3,500 x g for 10 min (4˚C) to eliminate contamination by cells or cellular debris from the serum. The pellet obtained was discarded and the supernatant was ultrafiltered through sterile 0.45 μm pore filters (Millipore, USA) in order to remove apoptotic debris and particles remaining in the supernatant from the first centrifugation. The supernatant was subsequently ultracentrifuged in microtubes (Hitachi No 1508) at 110,000 × g for 2 h at 4˚C in a Sorwal WX80 centrifuge with fixed-angle rotor (Fiberlite™ F50L-24 × 1.5). The resulting pellet was washed three times by ultracentrifugation in sterile PBS as described above and evaluated by transmission electron microscopy and NTA nanoparticle tracking analysis, as described in a previous paper [42].

For the isolation of DNA from EVs-DNA, the method described by Orrego et al., in 2020 [51] was followed. To do so, the sediment containing EVs from serum was treated with a total of 2 units of DNAase I at 37˚C for 30 min to remove external DNA from the EVs. After treatment, the enzyme was inactivated with heat at 70˚C in a solution containing 50 mM EDTA. After removal of free nucleic acids from the EVs suspension, 100 μL of EVs were lysed with 200 μL of lysis buffer (30 mMTris-HCl pH 8.0, 10 mM EDTA, 1% SDS) supplemented with 20 μL of proteinase K (0.1 mg/mL), shaken by vigorous pipetting and incubated at 56˚C for 1 h. Finally, nucleic acid purification and precipitation were performed following the traditional phenol-chloroform method by mixing 320 μL of lysed EVs suspension with 320 μL of phenol-chloroform-isoamyl reagent, 25:24:1 (v/v) (Sigma). The mixture was shaken briefly and centrifuged at 14,000 x g for 10 min. The aqueous phase was then collected and the DNA was precipitated with 1/10 vol of 3M sodium acetate pH 5 and 2.5 vol of HPLC grade absolute ethanol. The mixture was incubated overnight at -20˚C and centrifuged at 15,000 x g for 10 min at 4˚C, the supernatant was discarded and the precipitate was washed twice with cold 70% ethanol. Finally, the pellet was dried in a Jouan Thermo speed Vac (RC1010) at 20˚C and diluted in 20 μl Milli-Q water.

## Transmission electron microscopy

To corroborate by electron microscopy, the purification of EVs from serum, the ultracentrifuged pellet resulting from the purification process described above was fixed for 2h at room temperature in 2.5% glutaraldehyde in cacodylate buffer pH 7.2 and then resuspended in PBS for sample processing by negative staining. For this purpose, approximately 15 μL of the suspension were deposited on nickel 300 mesh grids, coated with a charcoal layer, for 10 min. The grids were then washed twice with ultrapure water for 1 min. The grids were negatively stained

with 1% uranyl acetate for 1 min. After staining, the samples were dried on filter paper and observed under a Zeiss Libra 120 Plus transmission electron microscope at 120KV at the Centro de Instrumentacio´n Cient´ıfica (University of Granada facilities).

## Detection of *T. cruzi* DNA in biological samples by different PCR strategies

**Genes used in the study.** In this work, two specific, conserved and highly repeated genes in the *T. cruzi* genome, widely used in the diagnosis of Chagas disease, were analyzed: the kinetoplast DNA (kDNA) minirepeat regions, which represent the major sequence component of kDNA with about 120,000 copies per parasite [52, 53], and the nuclear satellite (SAT DNA) with $10^4$ to $10^5$ copies in the highly conserved parasite genome [34].

## PCR strategies

**kDNA minirepeat regions and nested PCR.** DNA from the 448 serologically positive samples, extracted using the Maxwell Blood DNA Purification Kit automated protocol (Promega Biotech), and the minirepeated region of the kDNA was amplified. The reaction mixture was made up of Go Taq1flexi 1X amplification buffer (25 mM dNTPs, 2.5 mM $MgCl_2$, 1.0 U of Go Taq1(Promega, USA), 10 pmol of specific primers 121F and 122R (Table 1) and 10 μL of template. The final volume was 50 μL according to the methodology described previously [54]. Amplification conditions consisted of two cycles, each of 1 min at 98˚C and 2 min at 64˚C, followed by 33 cycles of 1 min at 94˚C, and 1 min at 64˚C, before a final extension at 72˚C for 10 min. In all cases, a DNA sample of *T. cruzi* from the culture as a positive control and a non-template control were used.

In all instances, the amplification result, although not visible on DNA electrophoresis, was subjected to nested PCR amplification. To this end, 2 μL of the PCR product were used as template, 0.2 μM concentration of T3 and T4 primers (Table 1), 0.2 mM of each dNTP, 3 mM $MgCL_2$, 0.5 μL of GoTaq Flexi DNA Polymerase; the final volume was 50 μL. The nested PCR conditions were (1) 94˚C 5 min, (2) (94˚C 1 min, 48˚C 1 min, 72˚C 1 min) × 35, (3) 72˚C 7 min, (4) 12˚C indefinite.

Amplifications were performed on a GeneAmp PCR System 9700 thermal cycler, AB Applied Biosystems and the nested PCR amplification product was sequenced on a GenomeLab GeXP system. The results obtained from sequencing were aligned using BLAST programs and the identity and coverage of these sequences were studied.

## EVs-DNA and cfDNA PCR

Both, the EVs-DNA present in the serum and the cfDNA were amplified indistinctly with the primer pair 121F-122R corresponding to the kDNA and with the SATF and SATR primers for

**Table 1. Sequences of primers used in this study.**

| Name | 5'!3' Sequence | Tm (˚C) | Type | Type (5'!3') | Product length (bp) | Gene |
|---|---|---|---|---|---|---|
| SATF | GCAGTCGGCKGATCGTTTTCG | 60.1 | PCR | F | 120 | Nuclear satellite DNA |
| SATR | TTCAGRGTTGTTTGGTGTCCAGTG | 58.5 | PCR | R | | |
| 121F | AAATAATGTACCGGKGAGATGCATGA | 65 | PCR | F | 330 | kinetoplastid DNA |
| 122R | GGTTCGATTGGGGTTGGTGTAATATA | 66 | PCR | R | | |
| T3 | TC TTT GGT GTG ATC GTT AC | 58 | nested PCR | F | 150 | kinetoplastid DNA |
| T4 | TAC ATT CTA TTT CTT CTC TG | 52 | nested PCR | R | | |
| Cruzi1 | ASTCGGCTGATCGTTTTCGA | 56.6 | Real time qPCR | F | | Nuclear satellite DNA |
| Cruzi2 | AATTCCTCCAAGCAGCGGATA | 56.4 | Real time qPCR | R | | |
| Cruzi3 | Fam-CACACACTGGACACCAA-NFQ-MGB | 52.2 | Real time qPCR | Probe | | |

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the nuclear satellite regions (Table 1) using for these amplifications 0, 5 μM of the primers, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.005 μL DMSO, 0.125 μL GoTaq Flexi DNA Polymerase enzyme and 2 μL as DNA template from DNA purification, the final reaction volume was 25 μL. For the SAT primers, the protocol was (1) 95˚C 5 min, (2) (94˚C 10 sec, 65˚C 10 sec, 72˚C 10 sec) × 40, (3) 72˚C 5 min, (4). Samples were kept at 12˚C.

The final products were visualized on 2% agarose gel stained with Safe Sybr and developed on a ChemiDoc MP Imaging System (Biorad). Electrophoresis was performed at 100 mV for 30 min.

### Nuclear satellite DNA real-time quantitative PCR

To ascertain if it is possible to quantify the DNA present in the EVs, the nuclear satellite sequence was amplified using real-time quantitative PCR (qPCR) following the validated protocol by Ram´ırez et at 2015 [18] with some modifications. The standard curve was generated using a starting quantity of 1.7 ng/μl from *trypomastigote stage of the parasite* DNA. The detection limit for DNA was determined to be 1.7 fg, with an efficiency of 101% and an R^2 value of 0.99. Standard curve (S1 Fig) and DNA-EVs samples were amplified according to the manufacturer's instructions for SsoAdvanced Universal Probes Supermix (BioRad 172–5281), using 700 nM of primers concentration and 200 nM of probe. The primers and probe sequences are shown in Table 1. DNA quantification was measured on light cycler Bio-Rad CFX96, cycling conditions (1) 95˚C for 10 min, (2) (95˚C for 15 sec, 58˚C for 1 min) × 39 cycles. All assays were performed in triplicate, including a template-free negative control and *T*. *cruzi* DNA positive controls prepared in a freshly prepared dilution series.

## Results

### Amplification of DNA from peripheral blood

The sera of 448 immunologically positive subjects, as indicated above, came from patients who had visited the different medical services of the Hospital La Fe. The majority (313) of whom were born in American countries, mostly in Bolivia. While 135, namely the youngest, were born in Spain but of Latin American mothers.

Fig 1A shows an example of 18 patients (15 positive and 3 negative) of the results of the first PCR with 330 bp amplicons. Nested PCR (corresponding to the kDNA) gave a positive result for 72 (16%). Eighteen samples are shown in which the 150 bp amplicon was visible in agarose electrophoresis (Fig 1B). Sequencing results of this amplicon are shown in Fig 1C. The results of the NCBI BLAST analysis of these sequences verified that the amplicon corresponded to a kDNA region of *T*. *cruzi*, 150 bp with coverage of 93% and an E of 2e-44 and an identity percentage of 97.4% (Fig 1C).

Analysis of the 72 samples, 448in which sample positivity was verified by nested PCR, resulted in 49 patients being female and 23 males. Mean age was 36.6 years (35 median) for females and 35.7 years (34 median) for males. Furthermore, positive patients were classified into 4 age ranges as shown in S2 Table. Thirty-four (5.6%) women were of childbearing age and 17 of whom were pregnant. Also, *T*. *cruzi* DNA was detected in the peripheral blood sample in the three children who were between 0 to 2 years old.

Most of the CD patients were born in Bolivia (n 70), one patient was from El Salvador and another one from Ecuador. S3 Table shows the distribution of origin in Bolivia, with Santa Cruz being the most represented region with 44.2% of the patients, followed by Sucre with 12.8%, Cochabamba with 10%, Potos´ı with 5.7%, La Paz (1.4%), Tarija (1.4%) and unidentified regions 25.2%.

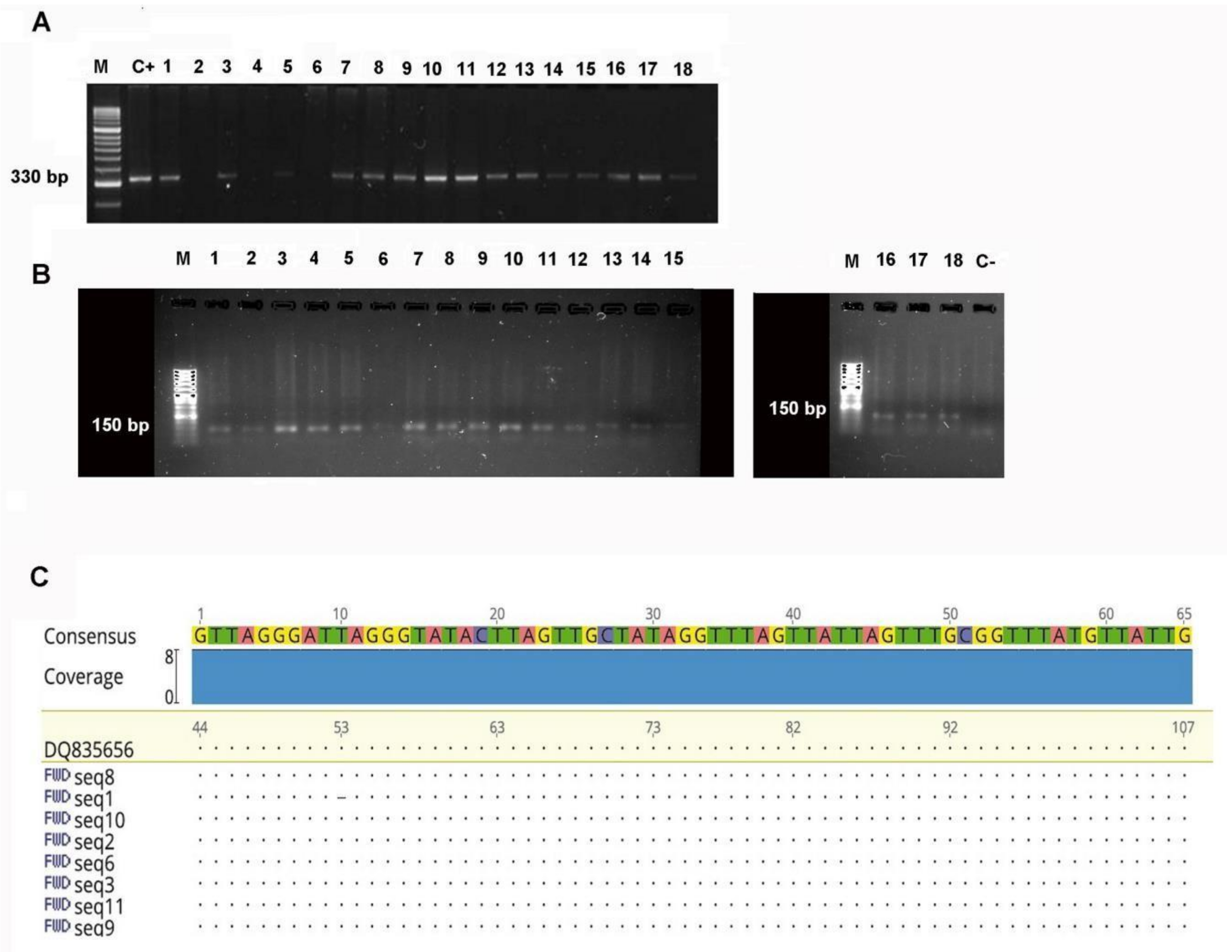


**Fig 1.** A: 2% agarose gel analysis of PCR-amplified products showing a 330 bp with 121 and 122 primers. PCR performed with peripheral blood DNA. Line 1, Hipper ladder II, Line 2, positive control, line 3 to 20 samples from 1 to 18 patients consecutively. B: 2% agarose gel showing a 150 bp band which belongs to nested PCR performed with T3 and T4 primers. C: Sequence alignment using the *T. cruzi* kDNA sequence with accession number DQ835656 as reference sequence. Our sequences were uploaded to GenBank with accession number as follow: OQ507819, OQ507820, OQ507821, OQ507822, OQ507823, OQ507824, OQ507825, OQ507826.

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S1 Table shows the conditions and age corresponding to the 72 positive nested PCR patients used in the study. Most of whom presented indeterminate symptoms (n 53), two had digestive disorders and nine patients suffered heart conditions. Of the total of 72 samples, 25 corresponded to patients who had not yet been treated with benznidazole. Also, it is noteworthy that of the 47 that had been treated only three remained PCR positive for *T. cruzi*. The period between the last dose of benznidazole and PCR was 1 year.

## DNA serum EVs and cfDNA amplification

**Confirmation of circulating serum EVs.** TEM visualization of EVs from serums pool isolated by ultracentrifugation is shown in Fig 2A. The mean size of the EVs was 119.92 ±18.41 nm. NTA analysis (Fig 2B) revealed the different populations of these EVs in which the majority peak revealed a size of 163 nm. A second population less numerous of EVs with an average

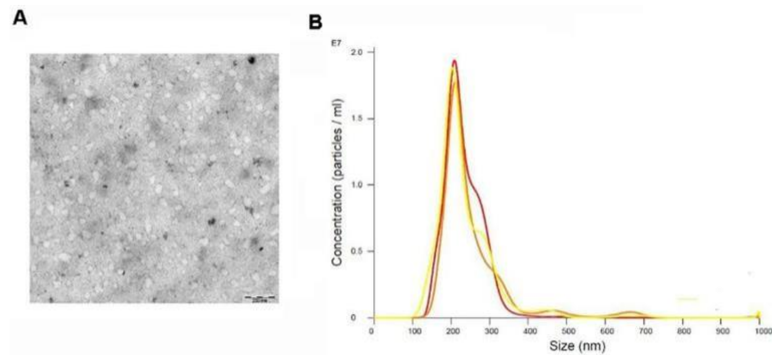


**Fig 2. EVs characterization.** A: isolated serum pool EVs observed under TEM. B; NTA showing the EVs populations. Isolation of extracellular vesicles by centrifugation filtration procedure from patients' sera: A) Negative staining observed under Transmission Electron Microscopy (TEM) of the EVs obtained from the pellet after the purification of the EVs from the serum. The measurements of the EVs were carried out using the Imaje J software. (Scale bar: 200 nm). Mean diameter 119 nm; B) Nanoparticle tracking analysis and size distribution of EVs (the largest peak of number of particles corresponding to a size of 231nm).

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size of 231 nm was observed. Analysis of the total population of EVs showed a mode size of 208±84.8 nm; the mode being 163 nm. Also, few populations of larger size were observed which could be considered aggregates of the EVs purified from the circulating EVs in the serum of the patients studied.

**Liquid biopsy.** Samples were randomly selected using a stratified procedure between the two populations. Twenty-five of these were from the group of patients who did not test positive by nested PCR, and 25 sera were from the group of patients positive by nested PCR. Serum EV DNA (EVs-DNA) and circulating free cellular DNA (cfDNA) were purified from all the selected samples.

To perform PCR, as indicated in the Material and Methods section, primers 121F-122R and those corresponding to SAT satellite DNA were used with the DNA purified by these methods.

**PCR analysis using DNA-EVs.** The results of parasite EVs-DNA circulating in serum showed that twenty-five patients were positive for primers 121F-122R (Fig 3A) and all but one (24) were positive for SAT primers (Fig 3B).

**PCR analysis using cfDNA.** Isolated cfDNA was amplified by PCR with SAT primers. Fig 3C shows six positive samples (S1 Table) corresponding to patients with cardiac, digestive and indeterminate conditions with CD. The same samples were negative when amplified by PCR with primers 121F-122R.

**EVs-DNA quantification.** The Limit of detection (LOD) and limit of quantification (LOQ) of EVs-DNA (Table 2) were calculated following the methods described by Ram´ırez et al., 2015 [18]. In total, six EVs-DNA samples were analyzed using qPCR, and two of them were quantified to be 1.9 fg and 2.35 fg, respectively. The remaining samples yielded positive results but could not be interpolated on the standard curve.

## Discussion

In the last decades, the distribution of CD has spread due to human migration for economic reasons from geographical areas of Central and South America, where hematophagous vector transmission is still the case, to non-endemic areas of North America, Europe, Japan or Australia. Spain, Italy and Switzerland are the three European countries [55] with highest levels of Latin American communities, especially Spain, related to their common language as well as historical and cultural links. The health authorities in Spain and Tuscany (Italy), areas with a

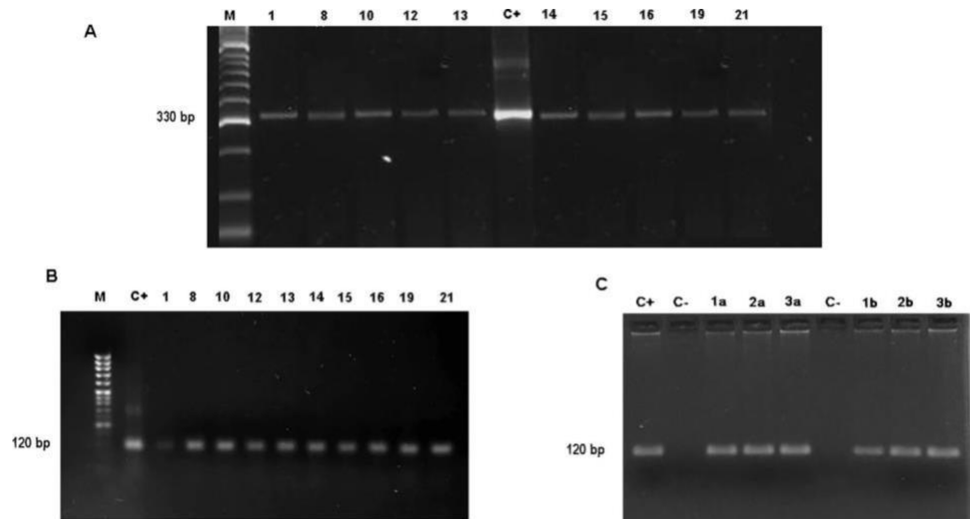


**Fig 3. 2% agarose gel electrophoresis analysis of PCR-amplified products.** A: PCR performed with 121 and 122 primers and EVs-DNA. Line M, Hipper ladder II; Line 6, C+, positive control; line 2 to 5 and lines 7 to 11 samples of patients. B: PCR performed with SAT primers and EVs-DNA. Line M, Marker, Line 2, positive control; line 3 to 10 samples 1, 8, 10, 12, 13, 14, 15, 16, 19, 21, respectively. C: PCR performed with SAT primers and cfDNA. Line C+, positive control; Line C- non-template control; Line 3 to 5 samples 1a, 2a, 3a; line 6 non-template control; line 7 to 9 samples 1b, 2b, 3b.

https://doi.org/10.1371/journal.pone.0282814.g003

high level of Latin American migrants have therefore adopted standards and recommendations on hemodonations [56], and transplant donors with regard to the detection of patients infected with *T. cruzi* in order to prevent human-to-human transmission of the disease [2, 57]. Similarly, regulations have been adopted for the screening of pregnant women originating from endemic countries and for which both the mother's and newborn's blood are tested [58, 59] in order to establish a treatment, which is well tolerated and effective in this type of patient. All this has prompted the standardization of diagnostic methods for CD in health centers and especially for chronically ill patients, in whom parasitemia levels are practically non-existent.

Classical parasitological diagnostic methods, such as visualization of trypomastigote forms in blood smears or thick drop, or even trypomastigote concentration techniques by centrifugation in microhematocrit tubes, have been used to diagnose CD [60, 61]. They show high specificity but very low sensitivity, especially in chronic patients. These methods may be applicable in diagnosis when the disease is developed with high parasitemia in the acute phase or after reactivation of circulating parasitemia in immunosuppressed chronic patients [60]. Only culture and xenodiagnosis can be used to directly detect *T. cruzi* infections in the chronic phase. These techniques are, however, not useful in clinical practice, especially in Europe, due to the limitations of insect vector handling and the delay in obtaining results, which limits their

**Table 2. Analysis of results obtained for qPCR assay.**

| Patient number | Mean ct | Limit of detection (LOD) (fg) | Limit of quantification (LOQ) (1.7 fg) |
|---|---|---|---|
| 8 | 31.5597125 | | 1.9 |
| 10 | 32.19097398 | 1.22 | |
| 12 | 32.99075464 | 0.69 | |
| 13 | 31.23063642 | | 2.35 |
| 14 | 32.83637534 | 0.9 | |
| 15 | 32.526172 | 0.95 | |

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usefulness in diagnostic studies and in evaluating treatment efficacy [62, 63]. Moreover, the evaluation of different techniques shows that in the case of low parasitemia, PCR is at least 27 times more sensitive than blood culture [64]. Also, Ramirez et al., 2015 applied an qPCR and a LOQ was estimated at 1.53 par. eq./mL and 0.90 par. eq./mL for SatDNA and kDNA respectively[18, 34]. These results were similar to the amount of *T*. *cruzi* specific DNA measured in our work (a LOD of 1.9 fg and a LOQ of 2.35 fg) when nuclear satellite DNA was quantified in EVs.

The course of the disease is characterized by elevated IgM levels during the initial acute phase of the disease and the appearance of IgGs that persist throughout the disease [65]. Thus, immunological diagnostic techniques are the gold standard for the diagnosis of CD in chronic patients, even in asymptomatic cases. The limiting factors in serological studies are the possible cross-reactivity with *Leishmania* spp. or *Trypanosoma rangeli* infections in those geographical areas where the disease was acquired and these trypanosomatids coexist [62, 66]. Another drawback could be the lack of sensitivity to the different parasite strains that affect humans throughout the Americas. They may present antigenic variability, which requires, if possible, the use of antigens of different geographical origin and different nature [24, 67] according to the recommendations of the different committees on CD. Individuals are considered infected when they have a positive result in at least two serological techniques using different antigens. A third technique is required in case of discrepancy of indeterminate immunological results [25–27, 68], or the use of a mixture of antigens that minimize discrepancies [22, 69]. The other significant case that invalidates immunological techniques is the diagnosis in newborns from infected mothers due to the presence of IgGs from the mother to the fetus by transplacental transfer [64, 70–72].

In cases that require the detection of the direct presence of the parasite in blood, when doubtful or inconsistent serological results are obtained and when the efficacy of the patient's treatment is evaluated, PCR methods capable of detecting the presence of parasite nucleic acids in blood [73] are needed to determine the unequivocal presence of the parasite. However, this technique, has a number of limitations that can alter the results and that must be considered [74, 75] such us the volume of blood processed, the correct handling of the blood (use of the chaotropic agent guanidine, time and treatment of the lysis process), the manual or automatic DNA extraction procedures, the presence and especially the elimination of PCR inhibitors, the primers used, etc. For all of these reasons, the quest for new procedures and methodologies in the extraction of nucleic acids to be applied to minimize false negative results continues.

The samples studied, reflect the Latin American population living and working in the area of influence of the Hospital La Fe in Valencia. Data from INE (National Institute of Statistics) for the year 2021 show that 17,847 people born in Bolivia reside in the Valencian Community, 39.1% are male and 60.9% are female.

In order to be able to select among the serologically positive patients those who could have parasitemia in the blood, and after carrying out the diagnostic PCR with the primer 121F-122R used in the hospital, and to increase the sensitivity of the detection, a nested PCR was performed on all the samples, in which the primers T3 and T4 designed for this purpose were used and which would amplify a band of 150 bp from the internal zone of the amplicon produced in the first PCR. The use of nested PCR using other genes with the products of the first PCR was already studied by Pereira et al., 2016 [76] and Ribeiro et al., 1999 [77], who concluded that the sensitivity of a series of 3 nested PCRs using primers specific for conserved regions of the Kinetoplast is able to detect 1 parasite per ml of blood, increasing the sensitivity of the PCR by about 10,000 times. Other authors such as Marcon et al., 2002 [50], or Riera et al., 2006 [78] employed nested PCR after conventional PCR using TCZ1 and TCZ2 and

internal primers TCZ3 and TCZ4 to evidence parasitization in a neonate. Also, Pereira et al., 2016 [76] used nested PCR for the products of a PCR using the TCZ1 and TCZ2 primers previously mentioned to detect CD positivity in a population of elderly people suffering from cardiac problems and in whom the involvement of *T*. *cruzi* in these pathologies was not suspected.

Sixteen percent of the total 448 immunologically positive patients tested positive in nested PCR.

The amplicons obtained were visualized by electrophoresis resulting in a band of 150 bp. Sequencing of the amplicons showed that they correspond to an internal region of the *T*. *cruzi* kDNA described by Burgos et al., 2009 [79], with a coverage of 93% and a percentage of identity of 97.4%, Fig 1.

The treatment of choice usually prescribed for CD-positive patients in Spain consists of 5 mg/kg benznidazole (BZ; N-benzyl-2-nitroimidazole acetamide) daily for 60 days. It is noteworthy that, of the 47 selected patients who had been treated, three tested positive post-treatment in nested PCR for *T*. *cruzi*. It was not ruled out that the treatment was not completed as a consequence of the side effects involved and the consequent interruption by the patient, and in one case the patient stated that he had returned to his country, not ruling out reinfection.

We also evaluated whether cfDNA present in the serum of CCD patients could be used as a template for specific PCRs to improve diagnosis in CCD and in neonates where parasitemia is very low and almost undetectable by other diagnostic procedures. The results of the analysis were 100% positive. This confirms that serum is a good sample to carry out DNA extraction to be used in the PCR detection of *T*. *cruzi*, which was already determined by Russomando et al., 1992 [80] and in the review carried out by Weerakoon et al., 2016 [81] and others [82, 83] where the use of cfDNA in different biological samples in the diagnosis of different parasitosis was encouraged.

In our study, patients who tested positive using EVs-DNA or cfDNA as PCR templates and patients who tested negative had similar characteristics; all of them had CCD. However, it is important to note that there was a higher proportion of positive patients who received no or incomplete treatment (7 of 25) compared with negative patients (4 of 25) (see S4 Table). Taking into account patient status, PCR was more successful in patients with known pathology compared to patients diagnosed as indeterminate (11 and 14 respectively for PCR positive vs. 4 and 18 for PCR negative). Of note, age and sex did not show significance within this population.

The results obtained in our cfDNA (nuclear DNA or mitochondrial KDNA) of parasitic origin would come from circulating parasites coming from either lysis or apoptosis of free or intracellular parasitic forms, or transported in elements secreted by the parasitic forms. With these sera we proceeded to the purification of EVs circulating in the serum, and the purification of the EVs from the sera of the patients was evidenced by both, electron microscopy and NTA. The term EVs designates the set of various EVs released by cells and delimited by a lipid bilayer that are released into the extracellular space by prokaryotic and eukaryotic cells [84].

EVs constitute cellular mechanisms of cell-to-cell communication over short or long distances between cells acting as endocrine, paracrine, juxtacrine or autocrine signaling [44], through uptake of EVs or receptor-mediated interactions. Evidently, blood is the mechanism of transport and diffusion of these EVs. EVs are classified by their size, biogenesis and composition, exosomes with sizes between 20 to 200 nm, ectosomes between 200 to 1000 nm and apoptotic vesicles of a larger size [41]. Exosomes contain functional molecules (including proteins, nucleic acids and lipids) derived from their cells of origin, being present in all biological fluids where they have been sought, urine and saliva and undoubtedly in blood [85–87]. Due to the protection of the lipid bilayer, exosomes are relatively stable and proteins and other

molecules transported in these small vesicles are protected from degradation. Their composition is quite complex, including a wide variety of lipids, proteins, different populations of RNAs, ssDNA, and metabolites [42]. Typically, the internal volume of an exosome ranges from 20 to 90 $nm^3$, suggesting that a prototypical exosome would contain approximately 100 proteins and 10,000 nucleotides [88].

In the present study, we have demonstrated the presence of parasite DNA, both from the kinetoplast and the nucleus. Furthermore, we have shown that it is possible to use it for diagnosis due to the high number of positively diagnosed patients, similar to what is found in experiments using total patient blood. The presence of parasite EVs in the serum of patients with CD was already described by some of us in 2017 [50] and these EVs appeared circulating in the serum forming immunocomplexes in patients with different Chagas pathologies, which denoted the presence of metabolically active forms of the parasite and capable of actively releasing these EVs. The fact that EVs capable of amplifying nuclear DNA and amplifying kDNA are found in the serum of those affected by CCD highlights the origin of circulating EVs, some originating from the endocytic pathway after fusion of late endosomes/mulvesicular bodies (MVB) of the parasite and the others of mitochondrial origin of the pathogen such as those recently described in the serum [89] and known as Mitovesicles, and involved in inflammatory processes [90].

It is the first time that this type of EVs carrying kDNA of strict mitochondrial origin has been described in the serum of patients affected by CCD, indicating the active presence of the parasite in the patients in whom it is found and constituting a marker for the presence of an active infection.

EVs have been used as liquid biopsy in numerous assays for tumor diagnosis [91–93], however, the use of EVs-DNA for the diagnosis of pathogens and especially intracellular pathogens has hardly been addressed so far. Cho et al., 2020 [94] showed that PCR using exosome DNA from isolates of patients affected by tuberculosis shows higher sensitivity than conventional PCR diagnosis using total DNA for PCR. To our knowledge, this is the first study to report the detection of *T. cruzi* DNA from circulating EVs in the serum of patients using two sets of primers, i.e., those that detect kinetoplast DNA minicircles (121F-122R) and those that recognize nuclear DNA with the SATF and SATR primers.

From the results of the present work, it is evident that both cfDNA and EVs-DNA from the serum of CCD patients constitute useful and effective biological samples to demonstrate by PCR the active presence of the parasite, in clinical cases (neonates and studies of the efficacy of treatment) in which it is necessary to demonstrate the presence of the parasite.

## Supporting information

**S1 Table. Patients information.**
(XLSX)

**S2 Table. Patients age ranges.**
(XLSX)

**S3 Table. Distribution of origin in Bolivia in regions.**
(XLSX)

**S4 Table. Positive patients and characteristics.**
(XLSX)

**S1 Fig. qPCR standard curve and samples.**
(TIF)

**S1 Raw images.**
(PDF)

## Author Contributions

**Conceptualization:** Mercedes Gomez Samblas, María José Giménez Martí, Maria Dolores Gómez Ruiz, M. Trelis, Antonio Osuna.

**Data curation:** Noelia Lozano, Mercedes Gomez Samblas, Antonio Osuna.

**Formal analysis:** Noelia Lozano, Mercedes Gomez Samblas, Eva Calabuig, María José Giménez Martí, Maria Dolores Gómez Ruiz, José Miguel Sahuquillo Arce, M. Trelis, Antonio Osuna.

**Funding acquisition:** Antonio Osuna.

**Investigation:** Noelia Lozano, Mercedes Gomez Samblas, María José Giménez Martí, Maria Dolores Gómez Ruiz, Antonio Osuna.

**Methodology:** Noelia Lozano, Mercedes Gomez Samblas, Eva Calabuig, María José Giménez Martí, Maria Dolores Gómez Ruiz, José Miguel Sahuquillo Arce, Sergio Sequera-Arquelladas, José Miguel Molina Moreno, M. Trelis, Antonio Osuna.

**Project administration:** Antonio Osuna.

**Supervision:** Mercedes Gomez Samblas, María José Giménez Martí, Maria Dolores Gómez Ruiz, M. Trelis, Antonio Osuna.

**Validation:** Mercedes Gomez Samblas, Eva Calabuig, María José Giménez Martí, Maria Dolores Gómez Ruiz, Sergio Sequera-Arquelladas, José Miguel Molina Moreno, M. Trelis, Antonio Osuna.

**Visualization:** Mercedes Gomez Samblas, Antonio Osuna.

**Writing – original draft:** Mercedes Gomez Samblas, M. Trelis, Antonio Osuna.

**Writing – review & editing:** Mercedes Gomez Samblas, Eva Calabuig, María José Giménez Martí, Maria Dolores Gómez Ruiz, José Miguel Sahuquillo Arce, José Miguel Molina Moreno, M. Trelis, Antonio Osuna.

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# SECCIÓN III: DISCUSIÓN Y CONCLUSIONES

# 5. Discusión

La EC crónica, como ya se ha comentado previamente, sigue siendo un gran reto para diagnosticarla de forma correcta en las áreas no endémicas. El principal objetivo de esta tesis surge de la necesidad de mejorar su diagnóstico, evaluando distintas técnicas diagnósticas realizadas por diferentes metodologías, i) estudio epidemiológico en el día a día de un hospital terciario, ii) estudio serológico utilizando las vesículas extracelulares como posibles marcadores, y iii) estudio molecular para detectar la parasitación activa.

La prevalencia de la EC, entorno al 12%, observada en nuestros resultados es similar a la de otros estudios realizados anteriormente (Romay-Barja et al., 2019; Velasco et al., 2020; Navarro et al., 2022). En España, la población latinoamericana encontrada con mayor prevalencia en los últimos artículos publicados (Herrero-Martínez et al., 2023) ha sido la boliviana, datos que se asemejan a nuestros estudios, ya que supone casi un 92% del total de la muestra estudiada.

El elevado número de reacciones adversas (RA), entre el 30% y el 87% de los pacientes, que presenta la administración de benznidazol como tratamiento de elección de la EC contribuye a unas tasas de abandono de hasta el 29% (Sperandio et al., 2014). Según nuestros resultados, un 49% de los pacientes inició la pauta de tratamiento con benznidazol y en un 54% de ellos desarrollaron reacciones adversas, lo que indujo unas tasas de abandono del 6%. La RA más comúnmente manifestada en estos pacientes fue la relacionada con síntomas cutáneos, datos similares a los descritos en la mayoría de los estudios publicados (Coronel et al., 2017).

En el subestudio de coinfección con *S. stercoralis,* se detectó una prevalencia del 12% en los 84 casos que se realizó la prueba. Este resultado avala, al igual que en otros estudios realizados previamente (Puerta-Alcalde et al., 2018), la necesidad de realizar un cribado de ambas patologías a todos los pacientes procedentes de áreas endémicas. La prueba de PCR anidada o *nested* PCR de *T. cruzi* se realizó a todas las muestras que tuvieron una serología positiva de *T. cruzi* en el hospital Universitario y Politécnico la Fe durante el periodo de

estudio. La justificación de las ventajas de realizar las PCR anidadas ya fue publicada en otros estudios como el de Ribeiro et al. (1999) y Pereira et al. (2016), quienes concluyeron que la sensibilidad de esta técnica aumenta hasta 10.000 veces más respecto a las diferentes PCRs sin reamplificar. Otros autores como Marcon et al. (2002) o Riera et al. (2006) aplicaron la *nested* PCR después de una PCR convencional para evidenciar la parasitación en neonatos. También Pereira et al. 2016, mencionado anteriormente, empleó la *nested* PCR para detectar la presencia de *T. cruzi* en la sangre de pacientes adultos con sintomatología cardíaca. De nuestros resultados se desprende que en el 20% de las muestras (85/433) con serología positiva se detectó la presencia de *T. cruzi* mediante la realización de la *nested* PCR, un porcentaje menor a lo publicado por Murcia et al. (2010) y la de Norman et al. (2011). Los amplicones obtenidos fueron visualizados por electroforesis en una banda de 150 pb, que, utilizando el método de secuenciación de Sanger, se observó que correspondían a la región interna del ADN del kinetoplasto de *T. cruzi* descrito por Burgos et al. (2009).

El diagnóstico de la EC activa sigue siendo una de las limitaciones para muchos clínicos e investigadores. La búsqueda de nuevos biomarcadores para identificar la presencia activa del parásito y el estado de la enfermedad continúa siendo un reto (Pérez- Molina and Molina., 2018; Kemmerling et al., 2019; de Sousa et al., 2024).

En este trabajo, se han aislado las EVs del parásito a partir del suero de pacientes formando inmunocomplejos. Se han comparado dos técnicas de purificación de EVs, la ultracentrifugación como "Gold standard" y la filtración por concentradores, como método más asequible y económico para ser usado en los laboratorios clínicos (Orrego et al., 2021). Tras la realización de este método, y como consecuencia de las propiedades fisicoquímicas de las membranas separadores, observamos, como inconveniente, una disminución de la concentración de las proteínas totales, respecto al "Gold standard" de la purificación de EVs. Estos filtros concentradores también han sido empleados en la búsqueda de las

vesículas extracelulares asociadas a otras patologías, como el cáncer o la enfermedad de Parkinson (Ayala et al., 2019; Vaswani et al., 2019; Orrego et al., 2021). Nuestros resultados indican que la decisión de utilizar una técnica u otra para aislar EVs circulantes en suero y determinar la presencia de material biológico de formas activas del parásito, depende del objetivo propuesto y de las instalaciones del laboratorio donde se realice la técnica. Este trabajo muestra que ambos métodos son comparables para demostrar la presencia de material circulante de origen parasitario en el suero o plasma de los pacientes con EC, implicando la presencia activa de *T. cruzi*. Por tanto, sería aplicable como método diagnostico para discriminar resultados no concordantes de ensayos inmunológicos, formas amastigotas quiescentes y la presencia de parásito en neonatos. Además, la pequeña muestra de suero requerida para realizar la técnica, lo convierte en más idóneo en el caso de los recién nacidos de madres con EC.

El bajo reconocimiento de los anticuerpos anti-MASP SP en las EVs del suero podría explicarse a partir de los hallazgos de Díaz-Lozano et al. (2017), quienes, mediante inmunocitoquímica por microscopia electrónica de transmisión, observaron que sólo el 45,19% de las EVs aisladas de los tripomastigotes procedentes de cultivos celulares mostraban marcaje. Esto indica que únicamente una fracción de estas EVs presentaba en su superficie las proteínas MASP inmaduras. Además, el número medio de partículas fue del 1,41 ± 0,65, lo que sugiere que sólo aproximadamente la mitad de estas EVs contenían los epítopos reconocidos del péptido altamente específico de *T. cruzi* correspondiente a las proteínas MASP inmaduras. Sin embargo, en el caso de los anticuerpos desarrollados frente a los antígenos totales de las EVs el reconocimiento fue mayor.

En nuestro estudio, la tasa de transmisión vertical observada fue cercana al 4% en recién nacidos de madres con EC, un valor similar al estimado por Carlier et al. (2019), quienes reportan una tasa de transmisión madre-feto de aproximadamente el 5%. En nuestro trabajo, se evaluó la presencia de EVs purificadas mediante ultracentrifugación a partir de

suero, utilizando un suero anti-EVs. Se analizaron muestras de 16 gestantes con enfermedad de Chagas, así como muestras de suero de sus hijos al mes y a los nueve meses de nacimiento. Siete de las gestantes habían recibido tratamiento con benznidazol antes del embarazo. Durante el seguimiento materno, cuatro de ellas presentaron PCR positiva para *T. cruzi;* sin embargo, solamente en un caso se confirmó la transmisión vertical de la enfermedad. En este caso particular, la lactancia materna fue suspendida al mes de vida del recién nacido, y tanto la madre como el lactante iniciaron tratamiento a partir del segundo mes.

En todos los casos, los valores de absorbancia obtenidos frente a las EVs procedentes de sueros mediante centrifugación, tanto de las madres como de los recién nacidos, fueron superiores que los controles negativos. En los recién nacidos, se observó un descenso de la absorbancia de anti-*T. cruzi* al mes y a los 9 meses del nacimiento comparándolo con las gestantes, excepto en los casos 7 y 8. El paciente 7 no acudió a la visita de seguimiento programada de los 9 meses. En el caso 10, la absorbancia de las EVs purificadas de la sangre del bebé, se vio ligeramente aumentada.

En el caso 16, el recién nacido que tenía una PCR de *T. cruzi* positiva al nacer, tras recibir tratamiento con benznidazol durante 2 meses, la absorbancia de las EVs a los 9 meses del nacimiento disminuyó hasta niveles prácticamente indetectables.

Durante un embarazo normal, la presencia de EVs en el sistema circulatorio del feto y la comunicación entre la madre y el feto se produce a través del intercambio de EV producidas tanto por la madre como por el feto (Sheller-Miller et al., 2019; Czernek et al., 2020). Las EVs derivadas de la placenta pueden representar un mecanismo mediante el cual la placenta se comunica para inducir adaptaciones en el embarazo. Estas EVs pueden servir de marcadores potenciales de diversas patologías fetales y maternas durante el embarazo (Adam et al., 2017; Buca et al., 2020; Nakahara et al., 2020). Las EVs obtenidas, tanto de la sangre de cordón umbilical, de la sangre neonatal o incluso de la orina del recién nacido,

sirven como marcadores de diversas patologías, particularmente en la prematuridad y durante el periodo de adaptación perinatal, desde el nacimiento hasta aproximadamente 4 semanas después del mismo (Murphy et al., 2021).

En los casos en los que se desee la detección directa del parásito en sangre, se hayan obtenidos resultados discordantes en la serología, o se requiera evidenciar la eficacia del tratamiento, se utilizarán los métodos moleculares. Sin embargo, estas técnicas tienen un gran número de limitaciones que pueden alterar el resultado (Junqueira et al., 1996; Diez et al., 2007; Alejando et al., 2018).

En cuanto al análisis de las diferentes subclases de IgGs que forman los inmunocomplejos en los pacientes con EC, observamos que, en los dos grupos de población estudiados, son las IgG2 e IgG4 las que presentaron mayores títulos de absorbancia. La determinación de los subtipos de inmunoglobulinas en la EC ha sido objeto de estudio por diferentes autores. Brodskyin et al. (1989) estudiaron las IgGs en la EC, sugiriendo que el aclaramiento inmunológico de *T. cruzi* es debido a los anticuerpos de los diferentes isotipos de IgG, particularmente en la subclase IgG2. Algo similar podemos observar en el estudio experimental llevado a cabo por Spynella et al. (1992), siendo la IgG2a la subclase que mayoritariamente se encontró, hasta 10 veces más que el punto de corte, especialmente en la fase crónica de la enfermedad. Esto podría indicarnos que algunos de los antígenos que portan las EVs, posiblemente glicosilados, estimularían la respuesta de anticuerpos desencadenando la respuesta por parte de las IgG2 e IgG4 por parte del hospedador.

Por todo lo que hemos expuesto anteriormente, respecto al bajo porcentaje de detección de actividad parasitaria en los pacientes con EC crónica y con la intención de poner a punto una técnica capaz de aumentar la sensibilidad en el diagnóstico neonatal, evaluamos el ADN libre en suero de estos pacientes. Los resultados obtenidos, confirmaron que las muestras del suero son adecuadas para llevar a cabo la extracción de ADN y realizar en ellas las técnicas de PCR para la detección de *T. cruzi*.

De los resultados obtenidos se puede deducir que el ADN libre de células es capaz de amplificar ADN de origen (nuclear o mitocondrial) del parásito. Dicho ADN podría proceder de parásitos circulantes, provenientes de la lisis o de la apoptosis de células libres o de formas parasitarias intracelulares y, lo más probable, transportadas en las vesículas secretadas por el parásito, dado que en el material de EVs purificados por centrifugación, aparece en dicha fracción. En esta fracción se evidenció la presencia de las EVs circulantes tanto por MET como por NTA.

Las EVs han sido usadas como biopsia líquida en numerosos ensayos relacionados con el diagnóstico de tumores (Inamdar et al., 2017; Luo et al., 2021; Wang et al., 2022). Sin embargo, el uso de ADN de las EVs para el diagnóstico de patógenos intracelulares fue descrito por Cho at al. 2020, mostrando que la PCR usando ADN procedente de EVs de pacientes con tuberculosis, mostraba una mayor sensibilidad que la PCR convencional. Nuestro trabajo es el primero en utilizar la EVs circulantes del suero o plasma de los pacientes con EC para la detección de la presencia de formas metabólicamente activas de *T. cruzi*. Los resultados de este trabajo evidencian que ambos, el ADN libre en suero y el ADN de EVs, dado que en el proceso se eliminó el ADN libre mediante DNAasa, procedente del suero de pacientes con EC constituyen una útil y eficaz herramienta para demostrar la presencia activa del parásito.

# 6. Conclusiones

Tras el análisis de todos los resultados, se han obtenido las siguientes conclusiones:

1. Los porcentajes de seroprevalencia obtenidos en este trabajo subrayan la importancia de perseverar en la vigilancia epidemiológica y el abordaje adecuado de esta patología en la población migrante latinoamericana, especialmente en aquellos provenientes de Bolivia.
2. Gracias a los protocolos de cribado neonatal de la enfermedad de Chagas, implementados en 2009 en el Hospital Universitario y Politécnico La Fe de Valencia, se ha podido determinar que los niveles de transmisión maternofetal de la enfermedad son similares a los valores descritos en otros estudios.
3. El bajo porcentaje de detección de *T. cruzi* por la técnica molecular de PCR, obtenido en los pacientes con enfermedad de Chagas crónica, pone de manifiesto la limitada efectividad de esta técnica en el diagnóstico o monitorización de dicha patología.
4. El uso de las vesículas extracelulares circulantes en el plasma o suero de los pacientes, que contienen material de origen parasitario, representa un método eficaz para determinar la presencia de formas metabólicamente activas del parásito.
5. La accesibilidad, el bajo requerimiento del equipamiento del laboratorio y el coste reducido del aislamiento de las vesículas extracelulares, mediante el uso de los concentradores de proteínas séricas, demuestran la gran utilidad de esta técnica para detectar las formas de parásitos activos, incluso cuando los niveles de parásitos en sangre sean muy bajos.
6. La utilización de técnicas moleculares como la PCR y la PCR cuantitativa (qPCR) de los ácidos nucleicos vehiculizados por las vesículas circulantes, muestra cómo dichas vesículas, portan ADN del Kinetoplasto o ADN de origen nuclear.
7. El uso de ADN libre de células presente en el suero o plasma (cfDNA) permite de

igual forma poder diagnosticar casos de la enfermedad de Chagas crónica y con baja o nula parasitemia.

8. Este trabajo pone de manifiesto la mayor presencia de las subclases de Inmunoglobulinas IgG2 e IgG4 en las vesículas extracelulares, formando inmunocomplejos circulantes en los pacientes con EC.

## Conclusión final

Esta tesis demuestra que, ante la necesidad actual de disponer de nuevos biomarcadores que discriminen la presencia de una parasitación activa en la enfermedad de Chagas, la detección de vesículas extracelulares circulantes en estos pacientes, mediante las técnicas moleculares descritas, contribuye al avance diagnóstico y seguimiento de esta enfermedad desatendida que afecta a tantos millones de personas. Por tanto, su uso puede resultar de utilidad en diferentes situaciones de incertidumbre clínica como la fase crónica de la enfermedad, la monitorización de la eficacia del tratamiento, la sospecha de formas parasitarias quiescentes y en casos de recién nacidos, donde las pruebas tradicionales de diagnóstico no son concluyentes e imposibilitan su tratamiento precoz.

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